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Pharmacogenetic studies of CYP2D6, CYP2C19 and CYP1A2, and investigation of their role in clinical response to antipsychotics and antidepressants

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PHARMACOGENETIC STUDIES OF CYP2D6, CYP2C19, AND CYP1A2, AND INVESTIGATION OF THEIR ROLE IN CLINICAL RESPONSE TO ANTIPSYCHOTICS AND ANTIDEPRESSANTS

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Thesis submitted in accordance with the regulations for the Degree of Doctor of Philosophy, University of London

December ~~2002~~ 2003

The work reported in this thesis was conducted in the:

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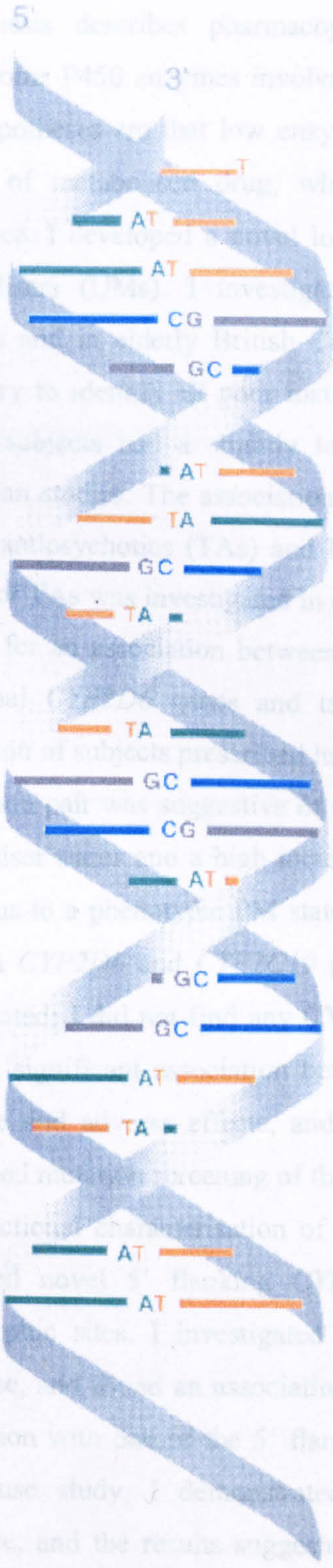
BRIT. LONDON.

To my husband, James,

without whose love and support this work would not have been completed,

and to my parents and sister, who each have a PhD, and whose example spurred me on

to finish mine.



Soli Deo Gloria

ABSTRACT

This thesis describes pharmacogenetic studies of CYP2D6, CYP2C19, and CYP1A2, cytochrome P450 enzymes involved in the metabolism of antipsychotics and antidepressants. The hypotheses are that low enzyme activity is associated with a high incidence of adverse effects of metabolised drug, while high enzyme activity is associated with therapeutic resistance. I developed a novel long-PCR assay for the identification of CYP2D6 ultrarapid metabolisers (UMs). I investigated CYP2D6 genotype-phenotype correlations in French subjects and in elderly British Caucasians, and found that genotyping for *CYP2D6*6* was necessary to identify all poor metabolisers (PMs) in French Caucasians, and that the elderly British subjects had a slightly lower mean CYP2D6 activity than the values in younger Caucasian studies. The association between CYP2D6 UM status and therapeutic resistance to typical antipsychotics (TAs) and between number of functional *CYP2D6* genes and adverse effects of TAs was investigated in case-control studies, which showed the surprising finding of a trend for an association between response to TAs and UM status, and between number of functional *CYP2D6* genes and tardive dyskinesia. This may reflect the relatively high proportion of subjects prescribed haloperidol in these studies. Conversely, a study in a sib pair and a twin pair was suggestive of an association between CYP2D6 poor or intermediate (IM) metaboliser status and a high incidence of adverse effects, and the conversion of a genotypic IM status to a phenotypic PM status through the prescription of thioridazine. The association between *CYP2D6* and *CYP2C19* gene dosage and response to tricyclic antidepressants was investigated: I did not find any CYP2D6 UMs amongst the treatment-refractory subjects, but found a significant association between CYP2D6 inhibition by concomitant medication and response and adverse effects, and between *CYP2C19* gene dosage and clinical response. I performed mutation screening of the *CYP1A2* 5' flanking region (identifying three novel SNPs) and functional characterisation of two of these, as well as sequencing of a BAC clone that generated novel 5' flanking *CYP1A2* sequence, including the identification of possible polymorphic sites. I investigated the association between *CYP1A2* SNPs and response to clozapine, and found an association between female gender and response, and a trend for an association with one of the 5' flanking SNPs that I had characterised. Finally, in a CYP1A2 null-mouse study, I demonstrated that CYP1A2 is the major determinant of clozapine clearance, and the results suggested that individuals with a relatively low CYP1A2 activity might well be more susceptible to adverse effects of clozapine including sedation and seizures. In conclusion, the activity of these polymorphic cytochromes would appear to influence the clinical response to metabolised drugs, but further larger prospective studies in this field are warranted, including the study of interactions between cytochrome P450 genotype and pharmacodyamic genetic factors (eg drug transporters and receptors), as well as open system pharmacogenomic approaches.

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ACKNOWLEDGEMENTS

I would like to thank the family and friends who have prayed and supported me through the hard graft of this work: my husband, my parents, my godmothers, Barbara, Beverley and Paul, Dominic and Naomi, Pat, Sue, Sally, Louise, church housegroups both in the UK and in the USA, the Maudsley and Institute of Psychiatry prayer group, and intercessors who support me and my husband. A special thank you goes to Barbara, who in addition has proofread this document.

I would like to thank the Wellcome Trust, who funded the majority of the work (in the form of a Mental Health Research Training Fellowship awarded to me), and the Royal College of Psychiatrists, for a Lilly Travelling Fellowship, that gave me the benefit of high quality experience in the laboratories of Drs Gonzalez and Quattrochi in the USA.

I would also like to thank my collaborators in this research, particularly Zing Hua Zhao, Pak Sham, and Kim Wolff for their assistance with the statistical analysis; Mahesh Patel and Michael Jann for analytical chemistry; Padraig Wright, Shubalade Smith, Janet Munro, Mark Kinirons, Catherine Bryant, Vivien Maskrey, Stuart Checkley, Michael Gill, Marc-Antoine Crocq, Luc-André Granier, Jean-Paul Macher, and Mark Taylor for their involvement in sample collections; Andrea Sapone, Guillermo Elizondo, Christoph Sachse, and Maria Arranz for assistance with laboratory methods; and Takafumi Sakai and Hani Zaher for training in animal handling. Special thanks go also to Kopal Tandon, and Evangelia Maria Tsapakis for carrying forward some of my studies and being such a joy to teach and work with, and to Jerson Pereira for his assistance with scanning in figures. And last, but not least, I would like to thank my supervisors: Dr AJ Makoff, Dr DA Collier, and Prof RW Kerwin.

STATEMENT CONCERNING CONJOINT WORK

CHAPTER THREE: PHARMACOGENETIC ASSOCIATION STUDIES

3.1 Genotype-phenotype correlation studies

3.1.2 French Caucasians

I performed all the DNA extractions and genotyping for *CYP2D6* alleles **3-5* for this study, in addition to developing the novel long-PCR assay for the identification of CYP2D6 ultrarapid metabolisers. For the latter, I received some assistance with primer design (from Alan G Gough), but developed the protocol and optimised the conditions unassisted, validating the assay with samples provided by Marja-Liisa Dahl (Sweden). The dextromethorphan phenotyping was performed in France by the collaborating group (Marc-Antoine Crocq, Luc-André Granier, and Jean-Paul Macher). Kopal Tandon performed *CYP2D6*6* genotyping on the two individuals with genotype-phemnotype discrepancy, under my supervision. I performed all the data analysis. Michael Gill was involved in a supervisory capacity in the early stages of this project, and latterly Robert W Kerwin, Andrew J Makoff, and David A Collier (RWK, AJM, and DAC).

3.1.3 UK Caucasians

I assisted in the design and collection of this sample, consenting the volunteers, collecting relevant clinical details, and scoring the General Health Questionnaire. I performed DNA

3.2.3 CYP2D6 UM status and failure to respond to TAs

I performed all the genotyping for this study, receiving only advice from Christoph Sachse regarding the conditions extractions, genotyping for *CYP2D6* alleles **3-5* and

CYP2D6 gene amplification. Kopal Tandon performed the *CYP2D6*2* genotyping and (on one sample) the *CYP2D6*6* genotyping under my supervision. The debrisoquine phenotyping was performed by Mahesh Patel at St Bartholomew's Hospital. I analysed all the data. Mark Kinirons and Vivien Maskrey from the Clinical Age Research Unit at King's College London were also involved in sample collection.

for one assay (the *CYP2D6*4 x 2* assay). Janet Munro, Padraig Wright and Shubalade Smith were involved in collection of the clinical sample and in clinical ratings. Pak C Sham provided some statistical advice.

3.2.4 Number of functional CYP2D6 genes and adverse effects of TAs

3.2.4.1 Sib pair and twin study

I performed clinical interviews, administered debrisoquine for phenotyping, and performed the genotyping for *CYP2D6* alleles **3-5*. Maria Arranz assisted in the early stages of optimising the *CYP2D6*4* assay. Kopal Tandon performed the *CYP2D6*2* and *CYP2D6*6* genotyping under my supervision. The phenotyping was again performed by Mahesh Patel. I analysed the data, identified genotype-phenotype discrepancies, and designed and supervised the subsequent work. Mark Taylor was involved in sample collection, and Robin M Murray in a supervisory capacity in addition to my three supervisors (RWK, AJM, and DAC).

3.2.4.2 Case-control study

I performed *CYP2D6*3-5* genotyping, *CYP2D6* gene amplification identification, and the data analysis, with some assistance in the statistical analysis from Jing Hua Zhao. Padraig Wright, Shubalade Smith, and Janet Munro were all involved in sample

collection, and Robin M Murray in a supervisory capacity in addition to RWK, AJM, and DAC.

3.3 Tricyclic antidepressants (TCAs) and CYP2D6 and CYP2C19

I collected the clinical samples, performed the clinical ratings (under the supervision of Stuart Checkley), and the genotyping for *CYP2D6* and *CYP2C19* variants in this study, other than the *CYP2D6*2* and *CYP2D6*6* genotyping, which was done by Kopal Tandon. The phenotyping was performed by Mahesh Patel (other than the phenotyping of two samples, which was performed by Gunnel Tybring's laboratory in Sweden) and I performed all the data analysis, with some statistical advice from Jing Hua Zhao.

3.4 Clozapine and CYP1A2

I developed the PCR-RFLP assay for the $T_{-3591}G$ SNP (which I had characterised, see below), and genotyped for this variant in the sample. Eva Tsapakis performed the CYP1A2 $C_{-164}A$ PCR-RFLP assay under my supervision. I conducted the data analysis, with some statistical assistance from Jing Hua Zhao. Janet Munro was involved in the sample collection.

CHAPTER FOUR: CYP1A2 NOVEL MUTATION SCREENING AND FUNCTIONAL CHARACTERISATION

I performed the genome walking, PCR sequencing, PCR-RFLP analysis, direct sequencing from a BAC clone, cloning of PCR products, site-directed mutagenesis, and transient transfections. Andrea Sapone assisted me in the early stages of the PCR sequencing, and Hani Zaher and Guillermo Elizondo were both involved in the

generation of the BAC clone. Catherine Bryant and Janet Munro were involved in the collection of clinical samples, and Frank J Gonzalez and Linda Quattrochi, were involved in a supervisory capacity.

CHAPTER FIVE: CLOZAPINE PHARMACOKINETICS AND PHARMACODYNAMICS STUDIED WITH CYP1A2-NULL MICE

I performed the pharmacokinetic study in the mice, optimising the protocol so that the clozapine dose and the timing of blood sampling was such that meaningful data would result. Takafumi Sakai and Hani Zaher taught me how to handle the mice, Michael W Jann performed the HPLC analysis, Kim Wolff and Jing Hua Zhao gave advice regarding data analysis, and Frank J Gonzalez was involved in a supervisory capacity.

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February 4, 2000

TO WHOM IT MAY CONCERN:

Dr. Katherine J. Aitchison spent nine months in my laboratory in 1998-1999, performing studies on CYP1A2. I was her direct supervisor, but her work was largely carried out independently. Her project was developed from her own ideas and used a number of resources available in my laboratory. Dr. Aitchison's research involved a number of techniques. These included PCR sequencing, genotyping using a PCR-RFLP assay that she developed, direct sequencing from a bacterial artificial chromosome (BAC) clone, and pharmacokinetic studies with clozapine using wild-type and CYP1A2-null mice. It should be noted that she performed all of the above techniques herself, having been assisted in the early stages of the PCR sequencing by Dr. Andrea Sapone, and having been taught how to handle the CYP1A2-null mice by Drs. Hani Zaher and Takafumi Sakai. Drs. Hani Zaher and Guillermo Elizondo performed the initial stages of screening a human BAC library in order to identify a CYP1A2-positive BAC clone and Dr. Aitchison confirmed the presence of the CYP1A2 gene by PCR and by direct sequencing of the BAC clone. During her studies in the NCI, Dr. Aitchison demonstrated a high level of skill in the laboratory and a keen insight into areas of research that are of great medical importance in drug therapy. I am convinced that she will be highly successful as an independent investigator at her current institution.

Sincerely,

Frank J. Gonzalez, Ph.D.
Chief, Laboratory of Metabolism

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315

January 27, 2000

To Whom It May Concern:

Dr Katherine J. Aitchison spent 4 months in my laboratory (from March 1999 to July 1999) performing studies on CYP1A2. Dr. Aitchison came to my laboratory to conduct studies of genetic mutations that she identified in the 5'flanking region of the human CYP1A2 gene. These studies comprised mutation characterization (including cloning), genotyping using a PCR-RFLP technique, site-directed mutagenesis, and functional studies using transient transfections. She performed all of these techniques herself, including optimization of conditions where necessary. The wild type construct (pL1A2N, containing the 5'flanking sequence of CYP1A2 proximal to a luciferase reporter gene) used for the transient transfections had already been created (Postlind et al., 1993), but site-directed mutagenesis was employed by Dr Aitchison to create the mutant constructs. Sequencing was performed by the Core Sequencing facility at the University of Colorado Health Sciences Center. I supervised Dr Aitchison's experimental work during her time in my laboratory; however, for the most part, Dr. Aitchison worked independently. I was quite impressed by how quickly she learned and implemented the advanced molecular techniques required for the success of this project. Her productivity during this relatively short time in my laboratory set an excellent standard for my lab personnel.

Sincerely,

Linda C. Quattrochi

Linda C. Quattrochi, Ph.D.
Associate Professor, Medicine
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ABBREVIATIONS

AIMS	Abnormal Involuntary Movements Scale
ANCOVA	Analysis of covariance
bd	twice daily
BNF	British National Formulary
bp	base pair
BSA	Bovine serum albumin
CARU	Clinical Age Research Unit
CG	Circle Grow (agar)
CYP1A2	cytochrome P450 1A2
CYP2C19	cytochrome P450 2C19
CYP2D6	cytochrome P450 2D6
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytosine 5'-triphosphate
df	degrees of freedom
DHPLC	denaturing high performance liquid chromatography
dGTP	deoxyguanidine 5'-triphosphate
df	degrees of freedom
DIP	drug-induced parkinsonism
DMF	demthylformamide
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
DSM-IIIR	Diagnostic and Statistical Manual of Mental Disorders, third edition, revised

dTTP	deoxythymidine 5'-triphosphate
EDTA	ethylenediamine teta-acetic acid
EM	extensive metaboliser
GAS	Global Assessment Scale
GHQ	General Health Questionnaire
GST	Glutathione *S*-transferase
HGMP	Human Genome Mapping Project
ICD-10	International Classification of Diseases-10, Classification of Mental and Behavioural Disorders
IM	intermediate metaboliser
IPTG	isopropyl-β-D-thiogalactoside
LB	Luria-Bertani (medium/agar)
Min	minute(s)
MR	metabolic ratio
NAT2	*N*-acetyltransferase 2
NIH	National Institutes of Health
PCR	polymerase chain reaction
PM	poor metaboliser
prn	as required
qds	four times daily
RDC	Research Diagnostic Criteria
RE	restriction endonuclease
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid

RT	room temperature
sd	standard deviation
SNP	single nucleotide polymorphism
SPSS	Statistical Package for the Social Sciences
TA	typical antipsychotic
TCA	tricyclic antidepressant
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TBE	Tris-borate EDTA
tds	three times daily
TE	Tris-EDTA buffer
TEMED	N,N,N',N'-tetraethylethylenediamine
Tris	Tris-(hydroxymethyl)aminomethane
TD	tardive dyskinesia
UDPGT	UDPglucuronyltransferase
UM	ultrarapid metaboliser
UV	ultraviolet (light)
WRSP	Webster's Rating Scale for parkinsonism
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside in DMF

CHAPTER ONE

PHARMACOGENETICS OF PSYCHOTROPIC DRUG METABOLISM

1.1 Definition of pharmacogenetics

The first major psychotropic agents to be introduced for the treatment of psychiatric disorders were lithium (Cade, 1949) and chlorpromazine (Delay *et al.*, 1952). Soon after their introduction, it became evident that there was marked interindividual variation in therapeutic doses of these, and although this was likely to be due to a complex interplay of multiple factors, it was proposed that genetic factors were partially responsible for this interindividual variation (Motulsky, 1957; Kalow, 1992). The study of the factors that were genetic in origin was defined as pharmacogenetics by Vogel (1959). The initial definition was relatively narrow: the study of heritable differences in the metabolism and activity of exogenous agents such as drugs or environmental toxins. More recently, this has been understood as comprising the study of variations in the coding or regulatory regions of genes that lead to interindividual variability in drug effect (efficacy) or in adverse effect profile (toxicity), but can also include benign differences such as the ability to taste certain chemicals (e.g. phenylthiourea, Kalow, 1992). Pharmacogenetics therefore includes the study of both pharmacodynamic genetic factors (affecting drug response at the site of action, *i.e.* target organ, *e.g.* at the level of receptor binding), and pharmacokinetic genetic factors (affecting drug absorption, metabolism, and excretion, *e.g.* cytochrome P450 enzymes in the liver) (Aitchison *et al.*, 2000c; Masellis *et al.*, 2000).

There are various clinical, behavioural, or physiological indicators of drug response in psychiatry at the level of the target organ, such as changes in severity of clinical symptoms as measured by standardised rating scales (e.g. the Hamilton Depression Rating Scale, Hamilton, 1967), changes in neuropsychological measures (e.g. deficits in working memory), or changes in endophenotypes, such as physiological gating mechanisms (e.g. pre-pulse inhibition). In animal studies, changes in behavioural responses or physiological gating mechanisms may be used.

In the US, in 1994, adverse drug reactions (ADRs) were between the fourth and sixth leading cause of death (ahead of pneumonia and diabetes mellitus), causing 106,000 deaths amongst hospitalised patients (Lazarou *et al.*, 1998). It is therefore important that advances in the field of pharmacogenetics are made in order to reduce the morbidity and mortality associated with ADRs. ADRs are also associated with prolongation of hospital stay (Chou *et al.*, 2000). Advances in pharmacogenetics and in the application of knowledge gained should have significant health economic consequences.

1.2 Evidence for heritability of drug effect

The first indication of heritability of drug effect was provided by Angst (1961), who observed concordance of response to imipramine among relative pairs with depression. This was soon followed by a more detailed report by Pare and colleagues (Pare *et al.*, 1962). They examined the records of 170 patients who had been treated with antidepressant drugs (imipramine or a monoamine oxidase inhibitor (MAOI)), mostly in the context of controlled trials. Twelve first-degree relatives had also been treated with a drug from one of these two classes. Treatment effects were concordant in all

proband-relative pairs (for response in 5 and non-response in 7). The authors also observed a tendency for the same patient to respond (or not respond) to a drug of the same class on repeated administrations (13 out of 17 patients consistent for MAOI and 7 out of 9 consistent for imipramine).

In 1967, Sjöqvist and colleagues observed a very large (36-fold) variation in the steady-state plasma concentrations of the tricyclic antidepressant (TCA) desipramine in a small group of patients on a standard dose (Sjöqvist *et al.*, 1967). The importance of genetic factors in determining the steady-state levels of the TCA nortriptyline was then shown in twin and family studies (Alexanderson *et al.*, 1969, 1973; Åsberg *et al.*, 1971a), and was similarly shown in a twin study of the kinetics of amobarbital (Endrenyi *et al.*, 1976).

In a new series of patients, Pare and Mack (1971) again observed concordance of response to antidepressants of the same class among first-degree relative pairs (10 out of 12 pairs). O'Reilly *et al.* (1994) reported a family multiply affected with major depressive disorder, four of whom did not respond to either TCAs or various new generation antidepressants, but did subsequently respond to the monoamine oxidase inhibitor, tranylcypromine.

Franchini *et al.* (1998) analysed 45 pairs of first-degree relatives consisting of a proband (with recurrent major depression or bipolar affective disorder) who had twice responded to fluvoxamine treatment of major depression and a relative who had been treated with the same drug. Thirty relative pairs (67%) were concordant for good response to fluvoxamine as compared to 50% expected by chance, with an excess of

bipolar patients among the relatives of subjects concordant for fluvoxamine response. The same group also described evidence for mendelian (oligogenic) mode of transmission of bipolar disorder in families with good fluvoxamine response (Serretti *et al.*, 1998).

A recent study of treatment-resistant depression in which data on various possible factors influencing response was collected noted that the only covariate that reached significance in terms of influencing whether an individual was responsive or resistant to antidepressant therapy was family history of affective disorder (Smith, personal communication).

With respect to antipsychotic response, Vojvoda *et al.* (1996) reported a set of monozygotic twins, each with a DSM-IV diagnosis of schizophrenia, who were concordant for treatment-refractoriness to various typical antipsychotic agents, but who had a positive response to clozapine. Furthermore, a heritable component to antipsychotic response has been demonstrated by studies with mouse strains (Dains *et al.*, 1996), and indicated by a human family study (Sautter *et al.*, 1993), and studies indicating differential response to antipsychotics in different ethnic groups (Frackiewicz *et al.*, 1997; Aitchison *et al*, 2000c).

There are a series of studies that have examined the role of genetic factors in response to lithium (for reviews see Alda, 1999; Alda, in press). Several (Mendlewicz *et al.*, 1973; Smeraldi *et al.*, 1984; Grof *et al.*, 1994), but not all (Coryell *et al.*, 2000), studies have found that a positive family history of bipolar disorder is associated with lithium response. Other studies have reported familial concordance of lithium response

(McKnew *et al.*, 1981; Grof *et al.*, 2000). Smeraldi *et al.* (1984) found evidence for a major gene effect in the families of responders, which was shown to be consistent with an autosomal recessive mode of inheritance by Alda and colleagues (1994, 1997).

There is, however, a relative paucity of studies investigating the heritability of drug response, and there are several reasons for this (Aitchison & Gill, 2002). Firstly, the phenotype to be measured is a transient, temporary trait, the response or adverse effects being elicited only in the presence of the drug. This means that it may well be difficult to find e.g. other family members or co-twins who are also taking the drug in question at the same time as the proband being investigated. If a family member has taken the same drug at some time in the past, it may be possible to perform a retrospective clinical assessment of their response or adverse effects, but this may be subject to recall bias or inaccuracy, etc. Secondly, the range of psychotropic medication available on the market has changed radically over the last 50 years, and continues to develop rapidly, so that what was available to a clinician treating a family member 30 years ago may be very different from the range of choices now available. Of note, the drug in the study of O'Reilly and colleagues (1994) was tranylcypromine, which due to its stimulant action is now regarded as the most hazardous of the monoamine oxidase inhibitors, and is rarely prescribed in the UK. Similarly, even if there are two or more family members simultaneously affected by a particular psychiatric disorder, the variety of available drugs for the treatment of psychiatric disorders means that the affected members may be prescribed different drugs, especially if they are geographically separated. Thirdly, in a study of families multiply affected with psychiatric illness, the precise disorder may vary amongst different family members, due to the polygenic nature of complex diseases, and the phenomena of incomplete

penetrance (Farmer *et al.*, 1987) and pleiotropy. Pleiotropy refers to genes segregating within a family, but apparently causing different phenotypes (e.g. bipolar disorder, depression, and schizophrenia segregating in the same family). These factors may clearly lead to variation in the psychotropic agents being employed. Fourthly, polypharmacy, i.e. the use of more than one psychotropic agent simultaneously (e.g. a mood stabiliser plus an antidepressant), is common these days, and this may lead to further difficulties in finding family members who are being treated only with a particular drug.

As good prospective data are hard to collect for the reasons outlined above, many pharmacogenetic studies to date have relied on retrospective collection of data regarding treatment history (including response and adverse effect profile to all current and previous agents used). From these, there is now a body of evidence indicating that genetic variation in activity of drug-metabolising enzymes has therapeutic and toxicological consequences (Bertilsson & Dahl, 1996; Bertilsson & Dahl, 1997; Ingelman-Sundberg *et al.*, 1999; Kalow, 1999, Wolf *et al.*, 2000; Ozdemir *et al.*, 2001; Kirchheiner *et al.*, 2001). Papers reviewed in the above are discussed in more detail in relevant sections of this thesis.

1.3 Phase I and phase II drug metabolism

Hepatic drug metabolism usually comprises two phases: phase I (oxidative metabolism), and phase II (conjugation). In phase I a metabolite with a functional group (e.g. a hydroxyl group) is formed, enabling the conjugation reaction in phase II to occur. The phase I reactions are mediated principally by the cytochrome P450 (CYP) enzymes, as well as others including the flavin monooxygenases (FMOs).

Several drugs (e.g. morphine) undergo direct phase II metabolism. Examples of phase II conjugation reactions include the addition of a glucuronic acid (by UDPglucuronyltransferases), a glutathione group (by glutathione *S*-transferases), or acetate (by *N*-acetyltransferases), or sulphate (by sulphotransferases). The phase I reactions in general take place in the endoplasmic reticulum, whilst most phase II reactions take place in the cytosol. This thesis focuses on phase I enzymes, notably three of the cytochrome P450s: CYP2D6, CYP2C19, and CYP1A2. I have also included CYP3A isoforms in this Introduction, as these are the most abundant hepatic isoforms, and hence impact upon metabolism through other routes, and other CYP2C isoforms, as the genes encoding these are in the same gene cluster as the gene encoding CYP2C19.

1.4 Cytochrome P450s

1.4.1 Characteristics of the superfamily

The CYP enzymes are haem-thiolate membrane-bound proteins (Omura & Sato, 1964a). The haem group is a protoporphyrin IX molecule and an iron atom, and acts as the prosthetic group for the CYP apoprotein. When the haem iron is in its reduced form (Fe^{2+}) and bound to carbon monoxide, and subjected to spectroscopy, maximal light absorption is seen at 450 nm – this feature is the basis for the P450 name and can also be used to measure the total CYP content of a given tissue in solution (Omura & Sato, 1964a, 1964b).

CYP nomenclature is based on degree of amino acid homology between different members of this superfamily of enzymes (Nelson *et al.*, 1996; Daly *et al.*, 1996a; see

also websites: http://www.imm.ki.se/CYPalleles; http://www.drnelson.utmem.edu/human.p450s). A CYP gene is named starting with the italicised *CYP*, followed by an Arabic number signifying the P450 family, an upper case letter signifying the gene subfamily, and another Arabic number for the individual gene (*e.g. CYP2D6*). The same sequence of letters and numbers is used in non-italicised form to represent the corresponding gene products (*i.e.* mRNA, protein, *etc.*). A P450 protein sequence from one gene family is defined as having ≤ 40% amino acid identity to a P450 protein from any other family, although there are exceptions to this (Nelson *et al.*, 1993). In a given family, there is >40% amino acid sequence homology between the members.

The extent of metabolism of a drug by a given CYP isoform is determined by both the affinity of the isoform for the substrate, and the relative abundance of the particular CYP in comparison to the other CYP isoforms in the tissue concerned. Shimada and colleagues (1994) estimated the relative content of various CYPs in human liver (Table 1.1), with 72% of the total CYP content being accounted for by the 7 isoforms or subfamilies studied. From this it can be seen that CYP2D6, for example, is a relatively low-abundance CYP in the liver. However, it has a relatively high affinity for a wide variety of substrates (both drugs and toxins, see below). By contrast, CYP3A is relatively high in abundance, but has a lower affinity for some of the CYP2D6 substrates. CYP2D6 therefore often functions as a high affinity, low capacity enzyme, which is more important at low substrate concentrations, while with higher doses or multiple dosing, enzymes such as CYP3A4, which is high capacity and may be low affinity, may play a more important role (Aitchison *et al.*, 2000c; Brøsen & Gram, 1998).

However, the study of Shimada and colleagues (1994) omitted some CYPs (*e.g.* CYP1A1), and, moreover, further CYPs have since been identified. These are likely to account for the remaining 28% unaccounted for in the original study.

Table 1.1 Relative content of individual CYP isoforms determined immunohistochemically in relation to total CYP content in human liver microsomes (values are means ± S.D. from 30 Japanese and 30 Caucasian liver samples, from Shimada *et al.*, 1994)

CYP isoform	**% of total CYP**
CYP1A2	12.7 ± 6.2
CYP2A6	4.0 ± 3.2
CYP2B6	0.2 ± 0.3
CYP2C	18.2 ± 6.7
CYP2D6	1.5 ± 1.3
CYP2E1	6.6 ± 2.9
CYP3A	28.8 ± 10.4

CYPs are also found extrahepatically. For example, CYP3A4, CYP3A5 and CYP1A2 are found in the intestine (Li *et al.*, 1998; Kuehl *et al.*, 2001). CYP1A1 is expressed in extrahepatic organs including prostate, mammary gland, intestine, thymus, colon, adrenal, lung, and testis (Shimada *et al.*, 1992, 1994, 1996; Ioannides *et al.*, 1990). CYP1B1, an enzyme with substrate specificity overlapping that of CYP1A1 and CYP1A2, but with different catalytic activity for these substrates (characteristic

substrate: 17βoestradiol), is expressed (at least at the level of mRNA) in many extrahepatic tissues including brain, heart, lung, intestine, testis, ovary, uterus, kidney, prostate, mammary gland, pituitary, thymus, spleen, and adrenal (Sutter *et al.*, 1994; Walker *et al.*, 1995), with particularly high levels in the kidney, prostate, uterus, and mammary gland (Shimada *et al.*, 1996). In addition to the liver and intestine, CYP3A5 is expressed in kidney, lung, and polymorphonuclear leucocytes (Kuehl *et al.*, 2001).

Studies of brain P450 have indicated that the total P450 abundance is approximately 1-10% of hepatic P450. Brain CYPs are implicated in local drug metabolism (e.g. it has been shown that rat brain 2D18 is capable of demethylating imipramine – Kawashima *et al.*, 1996; Thompson *et al.*, 1998), neurosteroid metabolism (notably CYP3A, 1B1, and CYP17 isoforms), neurotransmission (CYP2D6 is found in association with the dopamine transporter, and is inhibited by the dopamine reuptake inhibitor GBR-12935 – Britto & Wedlund, 1992; Niznik *et al.*, 1990 Hiroi *et al.*, 1997), and neurotoxicity (CYP1A2 is able to oxidise dopamine to aminochrome, which may lead to the formation of reactive intermediates, Segura-Aguilar, 1996; Segura-Aguilar *et al.*, 1997). However, although there have been some studies of human brain CYPs (Voirol *et al.*, 2000; Miksys *et al.*, 2002; for reviews see Kalow & Tyndale, 1992 and Ravindranath, 1998), the relative abundances, distributions, and substrate affinities of most brain CYPs remain to be defined. Furthermore the brain CYPs may differ from their hepatic counterparts: Thompson *et al.* (1998) reported novel cDNA clones isolated from rat brain which differed from the liver isoforms in their 5' and 3'-untranslated regions (UTRs). They commented that these differences could lead to differential catalytic activities, and, furthermore that this indicated differential regulation of CYP gene expression between liver and brain (*e.g.* rat 2D18, which has a

longer 3'-UTR than 2D4, is highly expressed in the brain, negligibly in the liver, Thompson *et al.*, 1998). It has been suggested that regional distribution of CYP isoforms in the brain may be of considerable relevance for the pharmacogenetics of psychotropic agents (Miksys *et al.*, 2000). Hedlund *et al.* (1996) showed that brain P450 was significantly induced by clozapine (especially 2D4), in terms of protein expression, but not by haloperidol.

Another interesting and relatively understudied facet of CYP genetics is developmental regulation of CYP gene expression. Multiple P450 forms have been isolated from liver microsomes of human foetuses, CYP3A7 being a foetus-specific and major form (Kitada *et al.*, 1985). A foetal CYP1A that differs from CYP1A2 has been purified (Kitada *et al.*, 1991); in the single neonatal liver sample examined by Shimada *et al.* (1994), a very low level of CYP1A2 was found, indicating that CYP1A2 expression may be increased developmentally (between the neonate and the adult). CYP1B1 was found in foetal heart, brain, lung, and kidney by Shimada *et al.* (1996).

1.4.2 Substrates of cytochrome P450s

The P450 gene superfamily is ancient, the ancestral gene having existed before the time of prokaryote/eukaryote divergence, with CYPs being implicated in basic life functions such as calcium ion and electrolyte balances, and cell division (Nebert, 1997). Drug metabolising enzymes (DMEs) including CYPs are involved in ligand-modulated regulation of transcription of genes effecting growth, differentiation, apoptosis, cellular homeostasis, and neuroendocrine functions (Nebert, 1994; Nebert, 1997).

The substrates of the CYPs are therefore manifold, including substances found naturally in the environment (xenobiotics) or the human body, with prescribed medications being only "fortuitously" metabolised. A summary of substrates of polymorphic human CYPs relevant to drug metabolism is provided in Table 1.2. Many enzymes show overlapping substrate specificity, and only the major routes of metabolism are shown. Substances may also exert considerable inhibitory effect at a given isoform, without being metabolised by that enzyme (*e.g.* quinidine in the case of CYP2D6).

1.5 Genetic variation in CYP activity

1.5.1 CYP2D6

1.5.1.1 Genetic polymorphism of CYP2D6

The debrisoquine/sparteine polymorphism was discovered independently by research teams led by R.L. Smith and M Eichelbaum in the late 1970's (Silas *et al.*, 1977; Idle *et al.*, 1978; Idle & Smith, 1979; Eichelbaum et al., 1979). Smith noticed that the antihypertensive agent debrisoquine caused an unexpectedly high incidence of side effects, and hypothesised that an underlying genetic variation in the way in which different individuals metabolised the drug might be responsible for the high incidence of undesirable responses. He and three laboratory colleagues took the standard prescribed dose, and measured the levels of metabolites in their urine. Smith became hypotensive and his urinary 4-hydroxy metabolite of debrisoquine was 18-fold less than that of his colleagues. They then screened a larger group of subjects, and poor metabolisers of debrisoquine, as they were termed, were found to represent 6% of a Caucasian population). Individuals were classed as either poor or extensive

Table 1.2 Some substrates of polymorphic CYP enzymes (from Aitchison *et al.*, 2000c; data from Bertilsson & Dahl, 1996; Schmider *et al.*, 1996; Andersson T, 1996)

CYP2D6		CYP3A4			CYP1A2	CYP2C19	CYP2C9
Haloperidol	Codeine	Haloperidol	Valproate	Oestradiol	Chlorpromazine	Clozapine	Amitriptyline
Perphenazine	Dextromethorphan	Clozapine	Codeine	Tamoxifen	Trifluoperazine	Olanzapine	Zopiclone
Zuclopenthixol	Methadone	Sertindole	Dextromethorphan	Progesterone	Clozapine	Amitriptyline	Theophylline
Thioridazine	Methamphetamine	Amitriptyline	Dextropropoxyphene	Oral contraceptives	Olanzapine	Imipramine	Phenytoin
Risperidone	Methylenedioxymethamphetamin*	Imipramine	Orphenadrine	Cortisol	Amitriptyline	Clomipramine	Tolbutamide
Sertindole	Propranolol	Clomipramine	Erythromycin	Prednisolone	Imipramine	Moclobemide	Warfarin
Amitriptyline	Metoprolol	Fluoxetine	Clarithromycin	Amiodarone	Clomipramine	Citalopram	
Clomipramine	Pindolol	Fluvoxamine	Doxycycline	Diltiazem	Zopiclone	Diazepam	
Imipramine	Timolol	Sertraline	Isoniazid	Nifedipine	Tacrine	Propranolol	
Desipramine	Flecainide	Nefazodone	Rifampicin	Nimodipine	Caffeine	Phenytoin	
Nortriptyline	Mexiletine	Trazodone	Trimethoprim	Nicardipine	Theophylline	Ibuprofen	
Fluvoxamine	Perhexiline	Venlaflaxine	Ketoconazole	Digitoxin	Aminophylline	Diclofenac	
Paroxetine	Propafenone	Diazepam	Itraconazole	Proguanil	Paracetamol/Acetaminophen	Naproxen	
Mianserin	Metoclopramide	Midazolam	Clotrimazole	Quinidine		Omeprazole	
Desmethylcitalopram	Orphenadrine	Clonazepam	Zidovudine	Cisapride		Pantoprazole	
Maprotiline	Ondansetron	Alprazolam	Ritanovir	Lidocaine		Proguanil	
Venlaflaxine	*also known as "ecstasy"	Zolpidem	Testosterone	Terfenadine		Piroxicam	
		Caffeine	Androsterone	Cyclosporin			
		Theophylline	Dapsone	Ondansetron			
		Carbamazepine	Dehydroepiandrostendione	Vinblastine			

metabolisers, based on their debrisoquine metabolic ratio (ratio of urinary debrisoquine to 4-hydroxydebrisoquine in an 8-hour urine sample following ingestion of the probe drug debrisoquine). Similarly, Eichelbaum and colleagues described the sparteine-N1-oxidation polymorphism, inherited in an autosomal recessive manner, and being controlled by "similar if not identical genetic factors" as the debrisoquine hydroxylation polymorphism (Eichelbaum et al., 1979; Bertilsson et al., 1980). Figure 1.1 shows the chemical structure of debrisoquine, and the site of the polymorphic 4-hydroxylation.

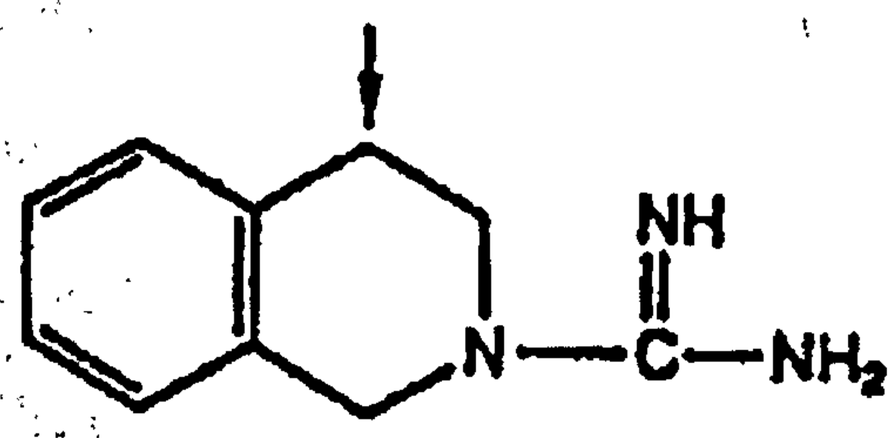

Figure 1.1 Chemical structure of debrisoquine; the arrow indicates the site of the polymorphic hydroxylation.

In a series of parallel investigations (Gough *et al.*, 1990; Hanioka *et al.*, 1990; Kagimoto *et al.*, 1990), a primary mutation at the DNA level associated with the poor metaboliser phenotype was described: a G1934A substitution at the 3' end of intron 3, resulting in an altered splice acceptor site, and hence a premature stop codon in the mRNA sequence, predicting a protein of 181 amino acids instead of 497. This turned out to be the commonest genetic variation in *CYP2D6* associated with the PM

phenotype (Heim & Meyer, 1990; Broly *et al.*, 1991; Dahl *et al.*, 1992; Marez *et al.*, 1997), and is now termed *CYP2D6*4*, although many others have subsequently been discovered. On the website where novel alleles are submitted, there are now 43 *CYP2D6* alleles, with several of these having subtypes (e.g. *CYP2D6*4A*, **4B*, **4C*, **4D*, **4E*, **4F*, **4G*, **4H*, **4J*, **4K*, **4L*; http://www.imm.ki.se/CYPalleles, searched 23rd July 2002, page updated by Mikael Oscarson 3rd July 2002). CYP2D6 now represents one of the most extensively studied monogenic variations in drug metabolism (Nebert, 1999; Sjöqvist, 1999).

CYP2D6 exists as part of a polymorphic gene cluster on chromosome 22q13.1, which includes two pseudogenes, followed by the coding *CYP2D6* sequence, oriented in the same direction 5' to 3' on a contiguous region of about 45 kb (Gonzalez *et al.*, 1988; Gough *et al.*, 1993; Kimura *et al.*, 1989; Gaedigk *et al.*, 1991; Heim & Meyer, 1992). The pseudogenes are *CYP2D8P* and *CYP2D7*, the latter also existing in variant forms (*CYP2D7AP* and *CYP2D7BP*). *CYP2D8P* is a pseudogene with many features typical of pseudogenes (Kimura *et al.*, 1989), while the key difference in *CYP2D7* as compared to *CYP2D6* is a T insertion at position 137 of exon 1, leading to a disrupted reading frame (Kimura *et al.*, 1989). Both pseudogenes show a high degree of homology with *CYP2D6*, the degree of homology between *CYP2D7* and *CYP2D6* obviously being greater than that between *CYP2D8P* and *CYP2D6*.

The multiple allelic variants in *CYP2D6* have been seen to correlate with four different levels of CYP2D6 activity: ultrarapid metabolisers (UM), extensive metabolisers (EM), intermediate metabolisers (IM), and poor metabolisers (PM) (Sachse *et al.*, 1997), which leads to a variation of up to 1000-fold in CYP2D6 activity (Bertilsson *et al.*,

1992; Meyer & Zanger, 1997). PMs, IMs, and UMs are defined below; the rest of the population are termed EMs.

Poor metabolisers are homozygous for non-functional *CYP2D6* alleles, *i.e.* alleles that do not give rise to the expression of any active CYP2D6 protein. Such alleles include *CYP2D6*3* (an A deletion at position 2637 leading to a disrupted reading frame; Kagimoto *et al.*, 1990), *CYP2D6*4* (described above), and *CYP2D6*5* (a complete deletion of the *CYP2D6* coding sequence, Gaedigk *et al.*, 1991; Steen *et al.*, 1995), which are the commonest non-functional *CYP2D6* alleles in Caucasians.

Intermediate metabolisers are either homozygous for alleles associated with reduced but not absent CYP2D6 activity (*e.g. CYP2D6*10* or *CYP2D6*17*), or have one reduced activity allele or one non-functional allele, or one reduced activity allele and one non-functional allele (Sachse *et al.*, 1997; 1998). The majority with a debrisoquine MR in the moderate range (3.0-12.6) fit into the latter category (Sachse *et al.*, 1998).

Ultrarapid metabolisers have extra copies of a functional *CYP2D6* gene in tandem due to gene amplification events (Johansson *et al.*, 1993; Johansson *et al.*, 1996), with between 2 and 13 copies of the gene being found in such individuals, most commonly 2 copies (gene duplication). Three individuals (members of the same family) with 13 copies of a functional *CYP2D6* gene have been described (Johansson *et al.*, 1993). Recently, it has been suggested that not all CYP2D6 UMs have gene duplications/amplifications, but that allelic variants could be associated with increased catalytic activity. Løvlie *et al.* (2001) suggested that *CYP2D6*35* could be associated with increased catalytic activity; however, functional studies of *CYP2D6*35* expressed

in yeast did not indicate increased catalytic activity as compared to wild-type enzyme (Allorge *et al.*, 2001). Nonetheless, the authors commented that the *CYP2D6* promoter polymorphism (-1584C>G) in linkage disequilibrium with the single nucleotide polymorphisms (SNPs) of *CYP2D6*35*, might possibly be associated with increased catalytic activity. Given the organisation of the *CYP2D* gene cluster, it has also been hypothesised that *CYP2D7* might be active in some individuals, and hence, in combination with an active *CYP2D6*, lead to an ultrarapid metaboliser phenotype (Løvlie *et al.*, 2001). However, Løvlie and colleagues (2001) sequenced *CYP2D7* exon 1 from 17 subjects selected for relatively low debrisoquine MRs (13 UMs and 4 EMs), who were negative for *CYP2D6* duplications, and all subjects were homozygous for the 137T insertion. Interestingly, *CYP2D7/CYP2D6* chimeric alleles (*CYP2D6*13* and *CYP2D6*16*, the former with exon 1 and part of intron 1 of *CYP2D7*, the rest of the gene originating from *CYP2D6*, and the latter with breakpoints between the end of exon 7 and the start of exon 9 in the respective genes, have been described (Panserat *et al.*, 1995; Daly *et al.*, 1996b).

The frequency of the different CYP2D6 allelic variants varies between different ethnic groups (Aitchison *et al.*, 2000c). In Caucasian populations, the frequency of PMs is 5-10% (Sachse *et al.*, 1997; Marez *et al.*, 1997), while in Black Africans the frequency is 0-8% (Woolhouse *et al.*, 1985; Masimirembwa *et al.*, 1996a, 1996b), in African-Americans the frequency is 3.7% (Leathart *et al.*, 1998), and in Orientals (Chinese, Japanese, and Koreans having been studied) the frequency is approximately 1% (Nakamura *et al.*, 1985; Lou *et al.*, 1987; Horai *et al.*, 1989; Sohn *et al.*, 1991; Du *et al.*, 1990; Bertilsson *et al.*, 1992). In addition, a lower population mean enzyme activity has been observed in Chinese, Zimbabweans, and Ghanaians as compared to

Caucasians. The low PM frequency in Orientals is caused mainly by the very low incidence of the *CYP2D6*4* mutant allele, the allele which is associated with absent enzyme activity and accounts for about 66% of PM alleles in Caucasians (Marez *et al.*, 1997). The lower population mean enzyme activity has been attributed to the relatively high frequency of *CYP2D6*10* in the Chinese, and of *CYP2D6*17* in the Ghanaians and Zimbabweans, both of which alleles being associated with diminished but not absent CYP2D6 activity, and known as intermediate metabolising alleles (Johansson *et al.*, 1994; Lee *et al.*, 1994; Masimirembwa *et al.*, 1996b; Droll *et al.*, 1998). The *CYP2D6*10* allele is a C188T substitution in exon 1, leading to a Pro34Ser change in a highly conserved region, and is associated with a 10-fold reduction in catalytic activity *in vivo* (Johansson *et al.*, 1994). The *CYP2D6*17* allele also occurs at a greater frequency in African-Americans (Leathart *et al.*, 1998). In addition, a further allele that occurs with relatively high frequency (20%) in Black populations has been identified: *CYP2D6*29* (Wennerholm *et al.*, 2001), which is associated with reduced catalytic activity for debrisoquine (63% of wild-type activity *in vitro*).

Dahl *et al.* (1995b) compared findings in a pilot study on Koreans, Chinese, and Japanese, and found that the frequency of *CYP2D6*10A* and *CYP2D6*10B* was somewhat lower among the Koreans than among the Chinese or Japanese. Therefore findings from one ethnic group may not be applicable to another geographically close and apparently similar ethnic group. Canadian Native Indians are descendants of North Asian populations, and have been found to resemble Chinese in terms of PM frequency, but to lack the shift towards a lower mean enzyme activity (Nowak *et al.*, 1997). This was seen to be due to a lower frequency of the *CYP2D6*3* and *CYP2D6*4* mutant alleles relative to Caucasians, and a lower frequency of *CYP2D6*10* compared with the

Chinese. Similarly, a relatively low frequency of *CYP2D6*10* has been found in the South-Amerindian population of Chile (Munoz *et al.*, 1998). This genetic drift has been interpreted as possibly due to a founder effect, mitochondrial DNA sequence variations revealing that these two groups of Amerindians were derived from a small number of maternal lineages (Bailliet *et al.*, 1994), or due to genetic selection pressures by dietary or other environmental factors. Middle Eastern populations show a very low frequency of CYP2D6 PMs, resembling Orientals rather than Caucasians in phenotyping studies (reviewed in Price Evans *et al.*, 1995).

A further factor to be considered amongst individuals of lower CYP2D6 activity is that in Black African populations, individuals who appear to be PMs when tested with one drug may not be PMs when tested with another drug, the two drugs both being metabolised similarly by CYP2D6 in Caucasians (Woolhouse *et al.*, 1985; Sommers *et al.*, 1989; Lennard *et al.*, 1992; Simooya *et al.*, 1993). This has been suggested to be due to either the presence of an as yet unidentified CYP2D6 variant with differential substrate specificity, or due to interethnic variations in conjugation and/or renal tubular transport.

At the other end of the spectrum of enzyme activity, the frequency of UMs also differs markedly between different ethnic groups, being 0.8-2% in Danes or Swedes (Dahl *et al.*, 1995a; Bathum *et al.*, 1998), 3.6% in Germans (Sachse *et al.*, 1997), less than 5% (Masimirembwa *et al.*, 1993; Masimirembwa *et al.*, 1996a) in Black Zimbabweans, 7% in Spaniards (Agúndez *et al.*, 1995), 20% in Saudi Arabians (McLellan *et al.*, 1997), and 29% in Ethiopians (Aklillu *et al.*, 1996).

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1.5.1.2 Clinical relevance of CYP2D6 polymorphism

Genotype-phenotype correlation studies indicate that the number of functional *CYP2D6* genes predicts drug and metabolite concentrations in the plasma: Dalén *et al.* (1998) showed that following a single oral dose of 25 mg nortriptyline to Caucasian volunteers known to have 0, 1, 2, 3, and 13 functional copies of CYP2D6, the concentration of nortriptyline was inversely related to the number of copies of CYP2D6, while the concentration of 10-hydroxynortriptyline (formed from nortriptyline in a reaction catalysed by CYP2D6) was directly related. The oral clearance of perphenazine and zuclopenthixol in patients on continuous treatment was also shown to be significantly predicted by CYP2D6 genotype (Jerling *et al.*, 1996).

It would therefore be intuitively logical that if PMs were at increased risk for drug toxicity during treatment with standard doses, then UMs might need doses higher than the normal therapeutic range in order to gain beneficial clinical effect. There is some evidence in support of this hypothesis, which is discussed in further detail in Chapter 3. For prodrugs that require activation by CYP2D6 (e.g. codeine), PM phenotype is associated with treatment resistance (Cleary *et al.*, 1994; Sindrup & Brøsen, 1995), and reduced capacity for codeine abuse. Chou *et al.* (2000) have estimated that the annual cost of treating patients at extremes of CYP2D6 activity (PMs and UMs) is a mean of 4000-6000 US dollars greater than for the CYP2D6 IMs and EMs, with the duration of hospital stay being prolonged for PMs.

There have also been studies of CYP2D6 and disease association, notably Parkinson's Disease and lung cancer. Meta-analysis of the literature is consistent with CYP2D6 PM status being associated with a small increase in risk of Parkinson's Disease (McCann *et*

al., 1997; Christensen *et al.*, 1998; Rostami-Hodjegan *et al.*, 1998). Subsequent studies have, however, yielded conflicting results, with positive associations being mainly found for the *CYP2D6*4* allele (Atkinson *et al.*, 1999; Bon *et al.*, 1999; Stefanovic *et al.*, 2000), although not invariably (Harhangi *et al.*, 2001), and not for other PM/IM alleles (Sabbagh *et al.*, 1999; Nicholl *et al.*, 1999; Joost *et al.*, 1999; Ho *et al.*, 1999; Maranganore *et al.*, 2000). It has been suggested that the apparent *CYP2D6*4* effect is an age-related artefactual association (Payami *et al.*, 2001).

Meta-analyses of the data for lung cancer indicate that the CYP2D6 PM phenotype is associated with a small decrease in the risk of lung cancer, which is not, however, held up in studies based on CYP2D6 genotyping (Rostami-Hodjegan *et al.*, 1998; Benhamou *et al.*, 1999).

There is some evidence that CYP2D6 PM status may be associated with anxious personality traits (Llerena *et al.*, 1993b; Peter Joyce, personal communication 2nd July 2002). Given the colocation with the dopamine transporter, there may be an as yet unidentified endogenous brain substrate for CYP2D6.

1.5.2 CYP2C enzymes

1.5.2.1 CYP2C genetics

Four members of the human CYP2C subfamily have been identified: CYP2C8, CYP2C9, CYP2C18, and CYP2C19 (Goldstein *et al.*, 1994); their genes form a cluster at chromosome 10q24. Of these, the role of CYP2C19 in the metabolism of

psychotropic drugs has been most extensively studied; relevant substances include: amitriptyline, imipramine, clomipramine, moclobemide, citalopram, diazepam, and desmethyldiazepam (Bertilsson & Dahl, 1996), as well as clozapine, olanzapine, propranolol, and phenytoin to lesser extents. Four SSRIs (fluoexetine, sertraline, paroxetine, and citalopram) all inhibit CYP2C19 and may also be metabolised by this enzyme. *CYP2C18* lies distal to *CYP2C19* on chromosome 10, is 85.7% homologous to *CYP2C19*, and shows similar substrate specificity towards diazepam (Jung *et al.*, 1997), phenytoin (Krecic *et al.*, 1995; Bajpai *et al.*, 1996), and omeprazole (Karam *et al.*, 1996). Furthermore, Mamiya *et al.* (1998) found cosegregation of poor metaboliser mutations of CYP2C19 and CYP2C18, indicating that CYP2C18 may not be able to take over from CYP2C19 in individuals deficient in CYP2C19. CYP2C9 was shown by Hashimoto and colleagues (1996) to play a greater role than CYP2C19 in the metabolism of phenytoin, and the Leu^{359} allele, which is present in the heterozygous state in 3.4% of Han Chinese subjects (Wang *et al.*, 1995), was seen to be associated with a 40% reduction in the V_{max} for phenytoin.

The incidence of poor metabolisers (PMs) of CYP2C19 in different populations has been reviewed (Price Evans *et al.*, 1995; Bertilsson, 1995; Persson *et al.*, 1996; Goldstein *et al.*, 1998). There is substantial interethnic variation: the frequency of PMs is 2-5% in Caucasians, 2% in Saudi Arabians, 4% in Black Zimbabweans, 5% in Ethiopians, 13% in Koreans, 15-17% in Chinese, 21% in Indians, and 18-23% in Japanese. Indeed, when the square root of the PM frequency (representing the total frequency of mutant *CYP2C19* alleles) is plotted versus longitude, an increase in this value versus longitude may be seen, with an increment in the value occurring between Saudi Arabia and Bombay (Price Evans *et al.*, 1995).

There are 2 wild-type *CYP2C19* alleles (*CYP2C19*1A* and *CYP2C19*1B*, i.e. with enzyme activities within the normal range), and seven defective alleles that are responsible for the PM phenotype (Goldstein *et al.*, 1998; http://www.imm.ki.se/CYPalleles, searched 23rd July 2002, page updated 15th February 2000 by Mikael Oscarson). The most common defective allele is *CYP2C19*2A* (a $G_{681}A$ substitution in exon 5, which creates an aberrant splice site, previous name for this allele, *m1*). A variant of this allele, *CYP2C19*2B*, contains a $G_{276}C$ substitution in exon 2 which creates a $Glu_{92}Asp$ change; this allele comprises 15% of the *CYP2C19*2* allele in Caucasians, but was not observed in 53 Japanese *CYP2C19*2* alleles studied. The two CYP2C19*2 alleles account for 86% of PM alleles in Caucasians and 69-87% in Orientals. The second major defective allele is *CYP2C19*3* (a $G_{636}A$ mutation in exon 4, which creates a premature stop codon, previous name for this allele, *m2*); this comprises 13-31% of PM alleles in Oriental populations and 1.5% in Caucasians. A third PM allele, *CYP2C19*4* (an A→G mutation in the initiation codon), accounts for 3% of Caucasian PM alleles. *CYP2C19*5* (a $C_{1297}T$ mutation in exon 9 which results in an $Arg_{433}Trp$ change in the haem binding region) accounts for 1.5% of Caucasian PM alleles and is rare in Orientals. *CYP2C19*6* (a $G_{395}A$ base substitution resulting in an $Arg_{132}Gln$ coding change in exon 3) and *CYP2C19*7* (a GT→GA mutation in the donor splice site of intron 5) each account for a further 1.5% of Caucasian PM alleles. *CYP2C19*8* (a $T_{358}C$ substitution resulting in a $Trp_{120}Arg$ change in exon 3) is a relatively recently characterised, rare defective allele. The products of *CYP2C19*6* and *CYP2C19*8* show reduced catalytic activity (2% and 9% of wild-type S-mephenytoin hydroxylase activity, respectively); the other mutants are associated with failure to express active CYP2C19. *CYP2C19*2A* and *CYP2C19*3* have both been identified in an Ethiopian

population, and found to account for all the PM alleles in the 114 individuals studied (Persson *et al.*, 1996).

1.5.2.2 Clinical studies of CYP2C19 and CYP2C9

Mephenytoin (Figure 1.2) and omeprazole have been used as probe drugs in studies of CYP2C19 activity. In single-dose studies, the clearance of omeprazole has been found to be higher in CYP2C19 EMs than PMs in Caucasians, Chinese, and Koreans, with the clearance in Caucasian EMs being significantly higher than that in both Chinese and Korean EMs (Bertilsson, 1995). After multiple doses of omeprazole, the mean areas under the plasma concentration-time curve for the parent drug indicated that heterozygous individuals had a reduced rate of metabolism as compared to homozygous EMs. It has therefore been hypothesised that the difference in clearance between Caucasians and Orientals is due to the relatively high proportion of heterozygous EMs among Orientals as compared with Caucasians.

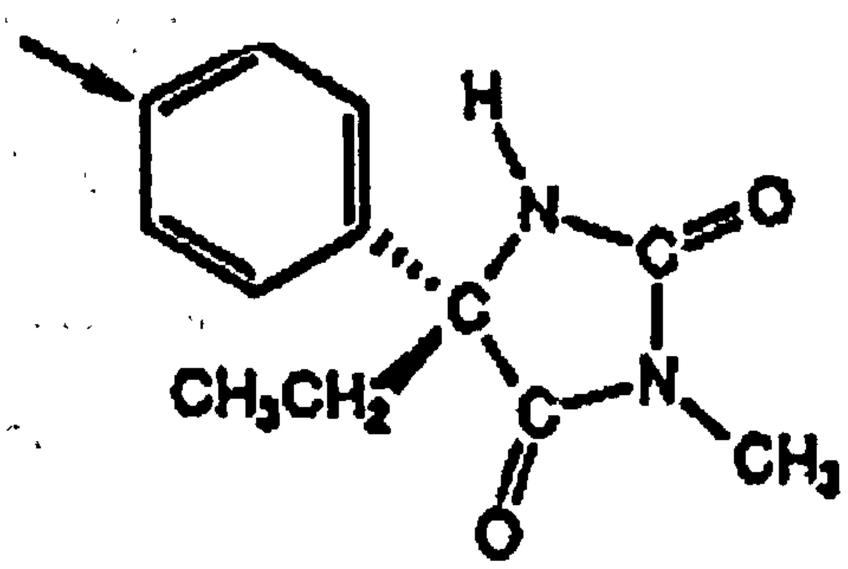


Figure 1.2 Chemical structure of mephenytoin; the arrow indicates the site of the polymorphic hydroxylation

In the case of diazepam, the clearance is significantly lower in Caucasian and Korean CYP2C19 PMs than EMs (Bertilsson, 1995). However, in Chinese, no significant difference between the elimination half-life of 8 EMs and 8 PMs was found, and the mean clearance in the whole group was relatively low as compared to Caucasians. It has been suggested that among the 8 Chinese EMs, 7 with a relatively low diazepam clearance might be heterozygous, which would explain the low overall clearance and the lack of significant difference between the EM and PM groups. Alternatively, differences in the contribution of CYP3A4 to diazepam pharmacokinetics in the different ethnic groups could explain the different findings. Like CYP2D6, CYP2C19 often functions as a high-affinity, relatively low capacity enzyme, which is more important at low drug doses. With higher doses, multiple-dosing, or in the case of CYP2C19 deficiency, the high capacity enzyme CYP3A4 (Table 1.1) increases in its contribution to overall drug clearance. Schmider *et al* (1996) have calculated that even with single doses, approximately 60% of diazepam clearance is CYP3A4-dependent. The relatively high incidence of low CYP3A4 activity in Chinese may therefore contribute to the low mean diazepam clearance, and, if polymorphisms in CYP3A4 and CYP2C19 do not cosegregate, could contribute towards the lack of a significant difference between diazepam clearance in S-mephenytoin PMs and EMs. It has been noted that "many Hong Kong physicians routinely prescribe smaller diazepam doses for Chinese than for white Caucasians" (Kumana *et al.*, 1987); this tradition is consistent with the lower clearance found experimentally.

An association between *CYP2C9* polymorphisms and warfarin dose requirement and risk of bleeding complications has been described (Aithal *et al.*, 1999). CYP2C9

catalyses the conversion of S-warfarin to inactive 6-hydroxy and 7-hydroxy metabolites, and there are 2 allelic variants, *CYP2C9*2* and *CYP2C9*3*, which show less than 5% and approximately 12% of wild-type CYP2C9 activity respectively (Rettie *et al.*, 1994; Haining *et al.*, 1996; Crespi *et al.*, 1997). Genotyping for the *CYP2C9*2* and *CYP2C9*3* alleles in a group of patients with a low warfarin dose requirement (1.5 mg or less), a group of randomly selected warfarin clinic controls, and 100 healthy population controls was conducted. This showed an odds ratio of 6.21 (95% confidence interval, or CI 2.48-15.6) for individuals with a low warfarin dose requirement having one or more CYP2C9 low activity alleles compared with the normal population. Patients in the low-dose group were more likely to have difficulties at the time of induction of warfarin therapy, including prolongation of length of inpatient admission when compared with the clinic control group (odds ratio 5.97, 95% CI 2.26-15.82), and had an increased risk of major bleeding complications when compared to this group (rate ratio 3.68, 95% CI 1.43-9.50). Pre-prescribing genotyping might therefore identify individuals more likely to have problems on induction, and lead to altered dose escalation procedures for these patients. In addition, the likely increased risk of major bleeding complications (in general later in the course of therapy) might lead either to increased monitoring of these clients, or a careful assessment of the risk-benefit ratio. The *CYP2C9*3* allele has also been shown to be associated with reduced clearance of phenytoin and tolbutamide (a hypoglycaemic agent) (Miners *et al.*, 1998).

1.5.3 CYP1A2

1.5.3.1 Variation in CYP1A2 activity

There is wide interindividual variation in CYP1A2 activity (Kalow and Tang, 1991a), which in most studies has been demonstrated to be trimodally or bimodally distributed (Butler *et al.*, 1992; Lang *et al.*, 1994; Nakajima *et al.*, 1994; Schrenk *et al.*, 1998; Ou-Yang *et al.*, 2000). This would appear to support the existence of genetic polymorphism in *CYP1A2*. Indeed, in a family study, the pattern of CYP1A2 activity in 8 pedigrees was consistent with genetic polymorphism at a single gene locus, with autosomal dominant transmission (Nakajima *et al.*, 1994).

At least some of the interindividual variability in CYP1A2 activity is, however, explicable by environmental factors. The enzyme is inducible by various dietary substances, drugs, and toxins. This includes cruciferous vegetables (*Cruciferae*, otherwise known as brassica, including broccoli, cauliflower, cabbage, watercress, and radishes), heterocyclic amines, polycyclic aromatic hydrocarbons (*e.g.* 3-methylcholanthrene), and heterocyclic aromatic hydrocarbons (including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, or TCDD), caffeine, cigarettes, paracetamol, omeprazole, and carbamezepine (Aitchison *et al.*, 2000a; Rost *et al.*, 1994; Parker *et al.*, 1998). CYP1A2 may be inhibited by lutein (found in leafy green vegetables, Le Marchand *et al.*, 1997), apiaceous vegetables (including parsnips, celery, and herbs such as dill and parsley; Lampe *et al.*, 2000), grapefruit juice (Fuhr *et al.*, 1993), oestrogens and pregnancy (Knutti *et al.*, 1981; Rietveld *et al.*, 1984; Abernethy and Todd, 1985; Vistisen *et al.*, 1992; Le Marchand *et al.*, 1997), quinolone antibiotics (Fuhr *et al.*, 1992), fluvoxamine (Brøsen *et al.*, 1993), and, in smokers, heavy ethanol consumption

(Rizzo *et al.*, 1997). Nonetheless, Le Marchand and colleagues (1997) showed in a study of 90 subjects of various ethnic backgrounds in Hawaii that 73% of the variance remained unexplained after taking into account the major environmental contributors to the variance. In a larger study (N=786 Caucasians), Tantcheva-Poór *et al.* (1999) found that 63% of the overall variation remained unaccounted for after analysis for the major covariates (*e.g.* coffee consumption). This finding points to the existence of other factors, such as genetic polymorphisms, as contributors to the variation in CYP1A2 activity. There is also evidence of interethnic variation in CYP1A2 activity (Section 4.1.2).

Nonetheless, the polymorphisms that have been identified to date in CYP1A2 would not appear to account for the apparent frequency of CYP1A2 poor metabolisers seen in the bimodal distributions. There are several possible reasons for this, including polymorphism in regulatory regions, and in other genes that may interact with CYP1A2. Indeed, in a study of caffeine *N*-3-demethylation (an index of CYP1A2 activity) in the mouse using quantitative trait methodology, Casley *et al.* (1999) showed that 62% of the variance was accounted for by 3 loci, on murine chromosomes 1, 4, and 9, the last colocalising with the murine *CYP1A2* locus. This points to the contribution of loci other than *CYP1A2* to indices of CYP1A2 activity, and is discussed in further detail in section 4.6.

1.5.3.2 Clinical relevance of variation in CYP1A2 activity

CYP1A2 plays a major role in the metabolism of many commonly used drugs, including chlorpromazine, trifluoperazine, clozapine, olanzapine, tertiary amine tricyclic antidepressants (amitriptyline, imipramine, and clomipramine), zopiclone,

tacrine, paracetamol, xanthines (caffeine, theophylline, aminophylline), and lignocaine (Imaoka *et al.*, 1990; Aitchison *et al.*, 2000c). It is also involved in the metabolism of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which produces a parkinsonian syndrome in man (Coleman *et al.*, 1996), and in the activation of arylamines and heterocyclic amines implicated in the genesis of colon and bladder cancer (McManus *et al.*, 1990; Boobis *et al.*, 1994; Eaton *et al.*, 1995; Hammons *et al.*, 1997).

There have been several *in vitro* studies investigating the role of CYPs in clozapine metabolism, which have indicated involvement of CYP1A2, but been inconsistent as to the relative importance of CYP1A2 and the precise role of CYP1A2 in the generation of the two major metabolites (Eiermann *et al.*, 1997; Linnet and Olesen, 1997; Tugnait *et al.*, 1999). This is discussed in further detail in Section 5.1.1. Interestingly, very low plasma clozapine levels despite high doses, in association with very high CYP1A2 activity, have been described (Bender *et al.*, 1998). It is therefore possible that, analogous to the variability of CYP2D6, there exist individuals with CYP1A2 ultrarapid metaboliser status.

The most important pathways for olanzapine metabolism are CYP1A2, flavin-containing monoxygenase 3, and N-glucuronidation, with minor pathways including CYP2D6 and CYP2C19 (Ereshefsky, 1996). Olanzapine clearance is increased in males (by about 30%) and in smokers, and decreased in the elderly, all of which are consistent with the involvement of CYP1A2. Evidence for the contribution of CYP1A2 to the pharmacokinetics of typical antipsychotics includes the effect of

smoking: smoking increases the clearance of fluphenazine and haloperidol by 100% and at least 50% respectively in an affected population.

1.5.4 CYP3A4

1.5.4.1 Variation in CYP3A activity

Human CYP3A activities reflect the heterogeneous expression of at least three CYP3A family members: CYP3A4, CYP3A5, and CYP3A7. The three genes coding for these enzymes are adjacent to each other on chromosome 7q21, together with two pseudogenes, *CYP3AP1* and *CYP3AP2* (Finta *et al.*, 2000, in the order *CYP3A4-CYP3AP2-CYP3A7-CYP3AP1-CYP3A5).* Functional CYP3A4 is found in most adults, with a 10- to 40-fold variation in its expression. CYP3A7 is predominantly expressed in foetal life; however, some people express CYP3A7 mRNA into adulthood (Schuetz *et al.*, 1994). Kuehl and colleagues (2001) have shown that CYP3A5 may comprise at least 50% of total hepatic CYP3A, in individuals that have at least one *CYP3A5*1* (wild-type) allele. There are two common allelic variants, *CYP3A5*3* and CYP3A5*6, which both cause alternative splicing and protein truncation, resulting in the absence of functional CYP3A5. Kuehl and colleagues (2001) also showed interethnic variation in expression of CYP3A5, CYP3A5 being expressed in 60% of the livers of African Americans, as compared with 33% of Caucasian livers.

CYP3A4 is present in the liver and small intestine, and can be induced, inhibited, or inactivated by drugs as well as environmental factors including food substances (Aitchison *et al.*, 2000c). Interpopulation variation in activity may therefore arise not

only secondary to intrinsic variation in enzyme activity, but also secondary to the effect of environmental agents.

Nifedipine is a cardiovascular drug that is metabolised by CYP3A4 and has been used as a probe drug to investigate CYP3A4 activity in different populations. It has been shown that South Asians (from the Indian subcontinent) oxidise nifedipine at a significantly slower rate than Caucasians (Ahsan *et al.*, 1991; Ahsan *et al.*, 1993), resulting in sustained haemodynamic changes. In the first study by Ahsan and colleagues, the South Asians had retained their original dietary practices, whereas the Caucasians consumed a typical Western diet. The effect of diet was studied in 6 Caucasians by giving them an Indian diet for 3 days prior to the administration of nifedipine; no significant difference in any of the pharmacokinetic parameters was detected. Similarly, the *N*-demethylation of codeine, which is catalysed by an enzyme of the CYP3A subfamily, occurs at a significantly slower rate in Chinese as compared to Caucasians (Yue *et al.*, 1989). Interestingly, Chinese have also been shown to have a significantly lower mean codeine *N*-demethylation activity as compared to Japanese (Yue *et al.*, 1995).

Twin and repeated drug administration studies have indicated that genetic factors play a prominent role in the regulation of CYP3A4 expression (Penno *et al.*, 1981; Ozdemir *et al.*, 2000). An A to G point mutation has been found in the nifedipine specific element (NFSE) at position -289 in the *CYP3A4* promoter (*CYP3A4*1B*, Rebbeck *et al.*, 1998), which has been shown to have a high frequency in African-Americans, and a low frequency in Caucasians and Taiwanese (Sata *et al.*, 2000). This allele was proposed to be associated with altered catalytic activity; however, functional studies performed by

Westlind *et al.* (1999) were not consistent with this. Kuehl *et al.* (2001) showed that there was linkage disequilibrium between *CYP3AP1*1* and *CYP3A4*1B* in African Americans, and as there was an association between *CYP3AP1*1* and high levels of CYP3A5 expression, postulated that it was *CYP3A5* genotype that was the major determinant of CYP3A functionality.

1.5.4.2 Clinical relevance of variation in CYP3A activity

CYP3A enzymes play a role in the metabolism of many typical antipsychotics, sertindole, and clozapine, in addition to many other prescribed medications and steroids (Table 1.2, and Ereshefsky, 1996). Drugs and food substances may act on the CYP3A4/5 present in the small intestine as well as that present in the liver. Indeed, the effect of a given agent on the hepatic and intestinal CYP3As may differ. For example, consumption of some furanocourmarins present in grapefruit juice can cause inactivation of enterocyte CYP3A4 while having no detectable effect on liver CYP3A4 activity (Watkins, 1998). Conversely, some oral drug regimens have been shown to increase liver CYP3A4 activity while having no effect on small bowel CYP3A4. It appears that some drugs (including benzodiazepines) undergo substantial first pass metabolism by enterocyte CYP3A.

Table 1.3 CYP3A inhibitors (modified from Aitchison *et al.*, 1999c)

SSRIs (fluoxetine, fluvoxamine), SNRIs (venlaflaxine, nefazodone)
Steroids (oral contraceptives, prednisolone, tamoxifen, *etc.*)
Antibiotics (erythromycin, troleandomycin, clarithromycin, isoniazid)
Antifungals (ketoconazole, itraconazole, clotrimazole)
AntiHIV drugs (ritonavir, zidovudine)
Analgesics (*e.g.* dextropropoxyphene)
Anaesthetics (*e.g.* midazolam, lidocaine)
Cardiac drugs (nifedipine, verapamil, diltiazem)
Immunosuppressants (cyclosporin, vinblastine)
Bromocriptine
Cimetidine

It has been shown that CYP3A4 is responsible for the back oxidation of reduced-haloperidol to haloperidol and also for the N-dealkylation of haloperidol (Pan *et al.*, 1998; Fang *et al.*, 1997). A negative correlation between clinical response and reduced-haloperidol levels or reduced-haloperidol/haloperidol ratios has been observed (Bareggi *et al.*, 1990); hence individuals with higher CYP3A4 activity could respond better to haloperidol than those with lower CYP3A4 activity. In a study on newly hospitalised Chinese patients with schizophrenia, Lane and colleagues found that those who experienced EPS had significantly higher reduced haloperidol concentrations and reduced haloperidol/haloperidol ratios than the other patients (Lane *et al.*, 1997). A

trend towards higher haloperidol concentrations was also found in the EPS group. This would be consistent with individuals with lower CYP3A4 activity being more vulnerable to EPS.

CYP3A4 is readily induced by carbamazepine; for most typical antipsychotics twice as much antipsychotic is required to achieve the same plasma concentration in the presence of carbamazepine as in the absence of carbamazepine (Ereshefsky, 1996). This interaction is relevant for the treatment of schizoaffective psychoses. Many other substances (mostly metabolised at least partially by CYP3A) inhibit metabolism by the CYP3A enzymes. Clozapine toxicity has been reported after the coadministration of one such inhibitor, erythromycin (Funderburg *et al.*, 1994). Individuals who are CYP2D6 poor metabolisers or who are in receipt of drugs that inhibit CYP2D6 metabolism would be expected to be at increased risk of effects secondary to drug interactions at CYP3A4, and *vice versa*.

1.6 Gene-environment interaction causing variation in CYP activity

There are several factors that may interact with genotype, increasing interindividual variation in CYP activity and leading to intraindividual variation in the same. These include drug-drug interactions, and the effects of dietary components or toxins (such as tobacco constituents), nutraceuticals, aging, and disease. Several have already been mentioned above, but will be covered in more detail below.

1.6.1 Drug-drug interactions

Interactions with other psychotropic medications and/or non-psychotropic medications may occur (Table 1.2), and have been extensively reviewed (Lin & Lu, 1998;

Greenblatt *et al.*, 1999; Fang & Gorrow, 1999; Tanaka & Hisawa, 1999; Flockhart & Oesterheld, 2000). Treatment with a potent CYP2D6 inhibitor (*e.g.* paroxetine or quinidine) may render an individual that is genotypically a CYP2D6 EM phenotypically a CYP2D6 PM. Indeed, even less potent CYP2D6 inhibitors, especially when administered chronically, may have this effect. Spina *et al.* (1991) showed that the proportion of subjects who were phenotypically CYP2D6 PMs increased on typical antipsychotic monotherapy, and Llerena *et al.* (1993a, 2001) showed that typical antipsychotic and antidepressant treatment was associated with CYP2D6 PM phenotype in individuals that were genotypically EMs. This effect may persist after cessation of administration of the relevant drug (depending on its half-life), and hence should be considered by prescribing clinicians when changing a patient's treatment regime (Alfaro *et al.*, 2000). It is possible to predict whether or not two drugs are likely to interact with each other at a given CYP if the following is known: a) which CYP isoform(s) are mainly responsible for the metabolism of the drugs b) the relative contribution of these CYPs to the total metabolism of the drugs c) the relative affinities of the drugs for these CYPs and d) the relative concentrations of the drugs in the tissue of interest (*e.g.* hepatocytes) (Andersson, 1996). If two drugs are significantly metabolised by the same CYP, they will often competitively inhibit each other, the metabolism of the drug with lower affinity for the enzyme being inhibited to a greater degree. Multiple CYPs may also be involved in such interactions, such that the net effect of the interactions may be difficult to predict, especially if there is induction of one CYP and inhibition of another. Many substances inhibit CYP3A enzymes at clinically used doses (Table 1.3). Coadministration of valproate and carbamazepine leads to increased formation of an epoxide metabolite of carbamazepine due to a complex interaction resulting from inhibition of CYP3A by valproate and induction of

CYP3A by carbamazepine. In such cases, phenotyping, if necessary using a cocktail of probe drugs to simultaneously evaluate multiple CYP activity (Streetman *et al.*, 2000), may yield data that are both clinically informative and useful for pharmacogenetics, as drug interaction effects may confound the interpretation of pharmacogenetics studies (Meyer *et al.*, 1996).

1.6.2 Nutraceutical effects

Many cultures have for centuries used herbal remedies and dietary constituents for the treatment of various conditions (*e.g.* Chinese herbal medicine; Milne, 1999), and there is now increasing interest in the Western world in these substances. However, it has become clear that these substances may affect the CYP system, leading to drug-drug interactions just like any other pharmacologically active agent.

For example, St John's Wort (extract of *Hypericum peforatum*) induces CYP3A, which may reduce the efficacy of protease inhibitors (e.g. indanavir, Piscitelli *et al.*, 2000), oral contraceptives (Ernst, 1999), and immunosuppressants such as cyclosporin (Mai *et al.*, 2000; Ruschitzka *et al.*, 2000). Possible mechanisms for this effect include enhanced transcriptional activity of the steroid X receptor, also known as the pregnane X receptor, a nuclear receptor involved in CYP3A induction (Wentworth *et al.*, 2000; Moore *et al.*, 2000), and increased P-glycoprotein activity (a transmembrane protein that extrudes drugs from cells and is responsible for resistance to drug treatment, encoded by the gene *MDR1*, multiple drug resistance gene 1; Johne *et al.*, 1999). Similarly, garlic supplementation may decrease the concentration of the antiviral saquinavir (a CYP3A and P-glycoprotein substrate, Piscitelli *et al.*, 2001), an effect that

may persist for more than 10 days after cessation of garlic supplementation taken twice daily for 3 weeks.

1.6.3 Dietary effects

Substances ingested as part of the diet without intention of any nutraceutical effect may also affect CYP activity. In addition to the effects already detailed, isothiocyanate-containing vegetables (*e.g.* watercress) can inhibit CYP2E1 (Kim & Wilkinson, 1996), organosulfur compounds present in garlic in animal model and *in vitro* studies inhibit CYP2E1 and induce CYP1A, CYP3A, and phase II enzymes (Brady *et al.*, 1991; Haber *et al.*, 1994; Kwak *et al.*, 1994, 1995; Reicks *et al.*, 1996), although this effect is unlikely to be relevant to the amounts of garlic or garlic products consumed in the diet (Wilkinson, 1997). Grapefruit juice components may inhibit and reduce intestinal CYP3A expression by selective post-translational downregulation of enterocyte CYP3A4 (Bailey *et al.*, 1998), and in some cases CYP1A (Bailey *et al.*, 1994; Dresser *et al.*, 2000), leading to large variation in bioavailability of CYP3A substrates. In addition, vitamins and spices have been implicated in altering drug metabolising enzyme (DME) activity (Wilkinson, 1997).

1.6.4 Influence of aging

From the third decade of life onwards, both liver blood flow and volume decline linearly over time, reducing to 20-40% of maximum by 80-90 years of age (Durnas *et al.*, 1990), accompanied by a reduction in clearance by the hepatic cytochromes CYP3A4/5, CYP1A2, CYP2C9, and CYP2C18/19 (Kinirons & Crome, 1997). For drugs that exhibit significant first-pass hepatic metabolism in young subjects, their

clearance in older subjects may be significantly affected by this phenomenon. There are no data on changes in intestinal CYP activity with aging.

1.6.5 Influence of disease

Acute inflammation, infection, and surgery may affect the metabolism of drugs and toxins (Wooles & Borzelleca, 1966; Renton & Mannering, 1976; Brockmeyer *et al.*, 1998; Shedlofsky *et al.*, 1994, Gidal *et al.*, 1996). The effects of inflammation or infection on CYP activity are thought to be due to stimulation of the cellular immune response, including via interleukins 1 and 6 and tumour necrosis factor (Renton, 2000; Watkins *et al.*, 1995; Haas, 2000). In HIV-infected individuals taking no medications known to alter DME activity, it was recently shown that CYP3A and CYP2D6 activities were reduced by 30-90%, with 25% of infected individuals showing genotype-phenotype discrepancy for CYP2D6 (Gotzkowsky *et al.*, 2000). This indicates that genotype-based pharmacogenetics assessments may have limited utility in HIV-infected individuals. Similarly, liver disease, especially cirrhosis, may affect DME activity (Vessell, 1984; George *et al.*, 1995; Wilkinson, 1997; Adedoyin *et al.*, 1998; Bechtel *et al.*, 2000).

1.7 Ethical considerations pertinent to pharmacogenetics

Ethical considerations pertinent to pharmacogenetics have been highlighted by Spallone and Wilkie (1999, 2000) and include the following (Aitchison & Gill, 2002). Firstly, what are the costs to the individual of population segmentation based on pharmacogenetic profile? For example, what might the psychological consequence be to someone to realise that they were excluded from a given treatment, e.g. clozapine, which is arguably the most effective antipsychotic to date? An individual might prefer

to take the risk of agranulocytosis and have haematological monitoring, depending on the magnitude of the risk, whereas a pharmaceutical company might not be prepared to take that risk: the risks acceptable to an individual and to a company might differ significantly. Similarly, to continue with this example, an effective treatment for agranulocytosis might be developed at some point in the future, and, if that were to happen, would the pharmaceutical company remove the exclusion from the particular population subgroup deemed to be at high risk of agranulocytosis, or would they consider that the possible associated risks or costs were for them still too high?

A related question is the likely size of the population subgroups resulting from population segmentation. The size of the groups resulting from a profile designed to predict drug efficacy are likely to be significantly larger than those designed to predict drug toxicity. Pharmaceutical companies might well be keen to identify relatively large subgroups likely to respond well to a given drug, but will it be cost-effective for a drug company to market a diagnostic test for a small percentage of individuals at risk of drug toxicity; what will be the threshold percentage, threshold cost of the test, and threshold severity of the potential ADR? With the economics of drug development already constrained to the point that many approved drugs never recover their development costs, it is unlikely that any pharmacogenetic strategies that require any significant increase in the scope or cost of drug development will be adopted by the industry. It has therefore been said that the challenge, then, for pharmacogenetics and pharmacogenomics is to invent and implement the novel technologies that can meet drug development's needs (Ledley, 1999).

Secondly, there are ethical considerations regarding informatics. There is the question of informed consent, when the current knowledge base regarding pharmacogenomics is relatively poor. What exactly does one tell the patient or volunteer that one is consenting for a study or clinical trial? To consent for a pharmacogenomics study requires a much broader consent provision than a pharmacogenetics study, the latter tending to involve specific candidate genes, the function of which one could make an attempt at explaining; the former requires consent for analysis of markers whose functional effect may not be known, and the possible consequences to the individual of having a particular known genetic marker profile in the future may be very difficult to predict. Some drug companies are making efforts to address these issues in written information provided to both investigators and patients in pharmacogenetics studies that are part of clinical trials (Spallone & Wilkie, 1999).

Then there is the question of protecting confidentiality for those about whom medical data is collected, relevant to any genetic study, and not unique to pharmacogenetics or pharmacogenomics; collaborations with pharmaceutical companies or biotechnology companies may render the issue particularly pertinent. There are three methods of handling data in order to enhance or ensure confidentiality (Spallone & Wilkie, 2000): anonymity, encoding, and encryption. Data is anonymous if all information capable of identifying the individual to whom the data relates has been removed and destroyed. Further information pertaining to that individual may then never be added to the appropriate record in the database. Data is encoded if a serial number or other code is attached to the data and a key to this is held elsewhere. Encoded data might be effectively anonymous to the research team working on it (e.g. laboratory research workers), if they did not have access to the key, but the data would not be truly

anonymous, as someone would be able to link the two. Encoding would allow updating of an individual's record, e.g. in the course of a longitudinal study. Encryption means turning the data into meaningless strings of numbers or letters, which are only decipherable by someone with the key. This latter method may be useful not so much for reasons of confidentiality towards the subject as for reasons of commercial security, to prevent unauthorised access by rivals to commercially significant collections of data. For pharmacogenetics and pharmacogenomics studies, in which it may be necessary to link detailed clinical information with genetic data, encoding would seem the most appropriate method of preserving subject confidentiality. This allows further clinical data to be incorporated in the database in time should research indicate that analysis with respect to other clinical variables might be informative. In the case of truly anonymised data, the sample can only be used once, then discarded, which may, of course, represent the loss of a potentially highly valuable genetic resource.

Finally, in terms of ethical considerations for pharmacogenetics/genomics, commercialisation, issue of intellectual property, and patent regimes for biotechnology may affect the doctor-patient relationship, which is vulnerable at a time when new partnerships developing between academia and industry may blur the boundaries between public and private interest. The involvement of industry in pharmacogenetics may constrain academic freedom, or restrict the use of any diagnostic tests resulting from the pharmacogenetics research, or restrict access to important resources including genetic sequencing databases and DNA banks. The latter has been a concern regarding, for example, the deCODE-sponsored databank in Iceland (Spallone & Wilkie, 2000).

All of the above should be considered when designing a pharmacogenetics study. All research reported in this thesis has been approved by the relevant Ethics Committees.

1.8 Aims of this thesis

This thesis describes pharmacogenetic studies of CYP2D6, CYP2C19, and CYP1A2, cytochrome P450 enzymes involved in the metabolism of antipsychotics and antidepressants. The hypotheses were that low enzyme activity would be associated with a high incidence of adverse effects of metabolised drug, while high enzyme activity would be associated with therapeutic resistance.

Chapter 3 describes a couple of genotype-phenotype correlation studies for CYP2D6, followed by a number of genetic association studies of CYP2D6, CYP2C19, and CYP1A2 and antidepressant and antipsychotic treatment response, including adverse effects. The aim of the first genotype-correlation study was to investigate the degree of genotype-phenotype correlation in French subjects, through genotyping for the *CYP2D6*3-5* and gene duplication alleles. The aim of the second genotype-phenotype correlation study was to investigate the degree of CYP2D6 genotype-phenotype correlation in an elderly sample, in order to ascertain whether due to age, a relative genotype-phenotype discrepancy developed.

The general aim of the genetic association studies was to determine whether or not there was an association between cytochrome P450 genotype or phenotype (specifically, that of CYP2D6, CYP2C19, or CYP1A2, or a combination thereof) and clinical response to treatment with antipsychotics and antidepressants. In the first reported genetic association study, I tested the hypothesis that patients with schizophrenia who were refractory to treatment with typical antipsychotics would be

more likely to be ultrarapid metabolisers, as compared with patients who responded to typical antipsychotics. In the next set of genetic association studies, I report investigation of putative association between CYP2D6 metaboliser status and susceptibility to adverse effects of antipsychotics. In a genetic association study of antidepressant treatment, there were several aims, including testing of the hypothesis that CYP2D6 ultrarapid metaboliser status would be associated with therapeutic resistance to treatment, that low CYP2D6 activity would be associated with a high incidence of adverse effects, and that CYP2C19 activity would also influence both clinical response and adverse effects. In the last genetic association study reported in Chapter 3, the aim was to investigate the association between *CYP1A2* polymorphism and response to clozapine.

Chapter 4 reports mutation screening and functional studies of the 5' flanking region of *CYP1A2*. Given the indication that factors including genetic polymorphism in *CYP1A2* could contribute to CYP1A2 variability and the negative finding of Nakajima *et al.* (1994), my aim was to screen the 5' flanking region of *CYP1A2* further upstream than –3470 bp,[1] to see whether or not a functional polymorphism, which could affect the inducibility of CYP1A2, could be found.

In Chapter 5, pharmacokinetic and pharmacodynamic studies of clozapine in CYP1A2-null mice are reported. The literature available at the time of this work was inconclusive regarding the contribution of CYP1A2 to clozapine metabolism. My aim was to use the CYP1A2 -/- mouse in order to investigate the *in vivo* contribution of CYP1A2 to clozapine pharmacokinetics. In addition, I used the CYP1A2 -/- mouse as a model for low CYP1A2 activity in man, through the use of behavioural ratings,

aiming to draw conclusions regarding the pharmacodynamic effects of clozapine in individuals relatively deficient in CYP1A2 activity.

CHAPTER TWO

MATERIALS AND METHODS

2.1 SAMPLES

2.1.1 Controls

2.1.1.1 French Caucasians / volunteers

A sample of 154 normal French male volunteers were recruited as part of a Phase II clinical trial (M-A Crocq and J-P Macher, Centre Hospitalier Specialisé, 68250 Rouffach, France). Forty-six also consented to phenotyping for CYP2D6 activity using dextromethorphan (see below).

2.1.1.2 UK Caucasians

A sample of 74 UK volunteers were recruited by the Clinical Age Research Unit, King's College London (S Jackson, M Kinirons, and C Bryant). Recruitment was from a variety of sources, including referral from medical practitioners, advertisement in local libraries and retired elderly people's day centres, and by word of mouth from existing volunteers. For each volunteer, the following information was ascertained: ethnicity, past psychiatric history, past medical history, alcohol intake, level of smoking, and current medication. In addition, the 28-item General Health Questionnaire (GHQ, Goldberg & Hillier, 1979, using the simpler "GHQ scoring method," 0-0-1-1) was administered. No volunteers gave a history of psychiatric illness and 4 had a GHQ greater than or equal to 5 (the threshold score 4/5 correlating with

psychiatric "caseness," Goldberg & Hillier, 1979). Forty-three of these volunteers were eligible for and consented to phenotyping with debrisoquine.

2.1.1.3 Taiwanese

One hundred and twenty-five normal Taiwanese volunteers (all Han Chinese) were recruited (J-D Huang, Department of Pharmacology, College of Medicine, National Cheng Kung University, Tainan, Taiwan), and blood samples were taken for DNA analysis.

2.1.1.4 Black Americans

One hundred and four American subjects of African descent were collected (W. E. Evans, Pharmaceutical Department, St Jude Children's Research Hospital, 332 North Lauderdale, Memphis, Tennessee 38101-0318, USA), and blood samples taken for DNA analysis.

2.1.2 Patients

2.1.2.1. Patients treated with typical antipsychotics

Sixty-six Caucasian subjects with DSM-IIIR schizophrenia were collected (P. Wright, South London and Maudsley NHS Trust). They were receiving treatment with typical antipsychotics (32% haloperidol decanoate, 25% fluphenazine decanoate, 22% flupenthixol decanoate) at doses equivalent to at least 100 mg chlorpromazine daily for at least 12 months prior to the date of assessment. All were examined for the presence of tardive dyskinesia (TD) using the Abnormal Involuntary Movements Scale (AIMS, US Department of Health, Education, and Welfare, 1974). A Research Diagnostic

Criteria (RDC) diagnosis of probable TD was made if subjects scored 2 or more in 2, or 3 or more in one of the 7 body areas assessed (Schooler and Kane, 1982). A further assessment using the AIMS was made between 2 and 6 weeks after the initial assessment, and persistent TD was confirmed if the above criteria were still fulfilled. In addition, all subjects were examined for the presence of drug-induced parkinsonism (DIP) using Webster's Rating Scale for Parkinsonism (WRSP). A diagnosis of DIP was made if a score of 2 (moderate slowness) or greater on the bradykinesia item, along with a combined score of 3 or greater on the rigidity and tremor items was reached (Metzer *et al.*, 1989). Six Caucasian patients with schizophrenia were also collected by S. Smith (South London and Maudsley NHS Trust), and rated for TD and DIP.

2.1.2.2. Affected twin pair and affected sib pair

One affected twin pair and one affected sib pair was collected (M. Taylor, R. M. Murray). The twins were 31 years at time of sampling, had a diagnosis of schizophrenia, and had both suffered adverse effects on a variety of antipsychotics (including extrapyramidal side effects or EPSE on typical antipsychotics, and neutropenia on clozapine). The sib pair was two brothers, 29 years and 23 years of age, both with schizophrenia, with an unaffected sister and unaffected parents. Both brothers had suffered EPSE on typical antipsychotics and neutropenia on risperidone. I administered the debrisoquine for phenotyping the twins, brothers, brothers' sister and parents (Lennard *et al.*, 1977), and took blood samples for DNA analysis. I also reviewed the medical records in order to ascertain a full medication history, and interviewed each subject.

2.1.2.3 Patients treated with clozapine

Two hundred and forty-six patients treated with clozapine in the UK were collected (R.W. Kerwin and J. Munro, South London and Maudsley NHS Trust). All of these patients were resistant to treatment with typical antipsychotics, and had a diagnosis of schizophrenia or schizoaffective disorder (clozapine prescribing restrictions in the UK, UK Clozaril Patient Monitoring Service). Prescribing consultants provided data regarding whether their patients were refractory to typical antipsychotics, intolerant of typical antipsychotics, or both refractory and intolerant of typical antipsychotics. Out of the 246 patients on clozapine, there were a total of 220 (89.4 %) who were refractory, 11 (4.5%) who were intolerant, and 15 (6.1%) who were refractory *and* intolerant to typical neuroleptics. The standard definition of treatment-refractory schizophrenia has been provided by Kane *et al.* (1988): (1) at least 3 periods of treatment in the preceding 5 years with antipsychotics (from at least 2 different chemical classes) at doses equivalent to or greater than 1000 mg/day of chlorpromazine for a period of 6 weeks, each without significant symptomatic relief, and (2) no period of good functioning within the preceding 5 years. Data regarding which typical antipsychotics had been prescribed were not available. However, the most commonly prescribed typical antipsychotic prior to switching to clozapine in the UK for the period during which our sample was collected was haloperidol (Novartis, personal communication). Seventy-nine were female (32.1%), one hundred and sixty-seven male (67.9%). The mean age was 39.0 years (range 19-81 years, SD 10.57).

In addition, information was collected from the psychiatrists prescribing clozapine regarding each patient's global level of response to clozapine, categorising the response into 1 of 4 categories: improved a lot (Category 1); improved a little (Category 2);

showed no improvement (Category 3); and inadequate information available (Category 4).

2.1.2.4 Patients treated with tricyclic antidepressants

I collected a sample of 49 patients with ICD-10 (World Health Organisation, 1992) depressive disorder (n = 41) or bipolar affective disorder (n = 8) in receipt of tricyclic antidepressant treatment, ascertaining the following information: ethnicity, age, alcohol intake (units/week), level of cigarette smoking (cigarettes per day), and current and previous treatment regime, and previous levels of tricyclics and their demethylated metabolites on previous treatment regimes. These patients were phenotyped for CYP2D6 status using the debrisoquine test (Lennard *et al.*, 1977, see below). I also performed Hamilton Depression Rating Scale ratings (HDRS, Hamilton, 1967; Guy, 1976), scoring patients prospectively for their severity of depression after a course of at least 6 weeks of tricyclic antidepressant (post-treatment score), and retrospectively for their pre-treatment score by interview at the post-treatment point with corroborative information from clinical records (I was trained in the use of the HDRS by Prof S Checkley). In addition, I rated the patients for adverse effects of the tricyclics using a modified version of the scales described by Åsberg *et al.* (1970) and Ziegler *et al.* (1978). Subjects were rated for the following adverse effects on a 0-3 severity scale (0 = absent, 1 = mild, 2 = moderate, 3 = severe): dry mouth, drowsiness, blurred vision, headache, constipation, nausea or vomiting, disturbance of micturition, interference with sexual function, palpitations, orthostatic symptoms, vertigo, sweating, tremor, disorientation, and weight gain. In their study of nortripytline plasma levels nad subjective side effects, Ziegler *et al.* (1978) found that eight of the above items (dry mouth, blurred vision, constipation, disturbance of micturition, orthostatic symptoms,

vertigo, sweating, and tremor) correlated more consistently with nortriptyline level, The 8-item corrected adverse effect score (total of score for these 8 items) was therefore also derived. Blood samples for DNA analysis and for serum levels of tricyclics and their *N*-demethylated metabolites were also taken.

2.1.3 Mice

A line of CYP1A2-null (-/-) mice were produced as described (Pineau *et al.*, 1995, Buters *et al.*, 1996), and were already available at the time of commencement of my study. In brief, a mouse CYP1A2 cDNA probe was used to isolate a 129/SV mouse genomic DNA clone, and disruption of CYP1A2 in this clone was generated by inserting the NEO gene (phosphoribosyltransferase II, confers resistance to the antibiotic G418) at a unique *Hind* III site in the second exon (first coding exon). A *Herpes simplex* virus thymidine kinase (HSV-TK) expression cassette was added to allow the use of ganciclovir for negative selection of homologous recombinant clones. Target vectors were linearised and used for electroporation into J1 embryonic stem cells (isogenic to 129/SV), cultured on γ-irradiated G418-resistant mouse primary fibroblasts, and G418 and ganciclovir resistant colonies were picked 6 days later. Restriction analysis and PCR were used to characterise positive clones and determine NEO orientation, and embryonic stem cells from one clone were injected in to the blastocoel cavity of 3.5-day C57BL/6N embryos. Embryos were transferred into pseudopregnant NIH Swiss foster mothers. Chimeric males were generated that gave germ-line transmission of the targeted allele. Homozygous mutant mice (-/-) were obtained by heterozygous (+/-) matings. Animal genotyping was carried out by Southern blot analysis of tail DNA.

In my study, I used wild-type mice of the same (129/SV) genetic background as the CYP1A2 -/- mice. These two strains have been previously characterised in detail by Buters *et al.* (1996), who showed that all parameters that might affect pharmacokinetic studies were not significantly different in the two strains. Specifically, they showed that renal function (as measured by creatinine), liver function (bilirubin, alanine transaminase, aspartate transaminase), protein binding (albumin), and the expression of other cytochrome P450s did not differ between the 2 strains.

All mice were kept in a barrier facility with 2 ppm chlorinated water, autoclaved food, and bedding under a 12h light-dark cycle, 40% humidity, with free access to food and water. The animals were fasted for 12 hours prior to dosing and for the duration of the experiment, but allowed water *ad libitum* throughout. I conducted pilot studies in order to determine the dose of clozapine that would produce detectable pharmacokinetic data without inducing toxicity in the mice, and also the time points at which samples should be taken for meaningful pharmacokinetic data to result. For this purpose, a total of 28 wild type mice and 19 CYP1A2 -/- mice in a total of 11 pilot studies were used, employing doses of clozapine ranging between 5 and 25 mg/kg, and taking blood samples at various time points up to 480 min post injection of clozapine. For the final experiment, four male CYP1A2 -/- and four male wild-type animals were used. These 8 mice had all been born 81 days prior to the day of the pharmacokinetic study.

2.1.4 Ethical Committee approval

All studies involving human subjects were approved by the South London and Maudsley NHS Trust (Bethlem and Maudsley Hospitals) and Institute of Psychiatry

Ethical Committee (Research), or local ethics committees, as appropriate. The mice study protocol was approved by the Animal Care and Use Committee of the National Cancer Institute (National Institutes of Health, Bethesda, MD, USA).

2.2 LABORATORY METHODS

The composition of buffers and other experimental reagents is described in Table 2.1.

2.2.1 CYP2D6 phenotyping

2.2.1.1 Debrisoquine phenotyping

Debrisoquine is an antihypertensive agent which is no longer licensed for prescription, but which has been well described as a CYP2D6 probe, CYP2D6 catalysing the formation of 4-hydroxydebrisoquine *in vivo* (Evans *et al.*, 1980). For debrisoquine phenotyping, subjects were instructed to empty their bladder, take a 10 mg dose of debrisoquine (Declinax ®, Roche Products Ltd.) before retiring, then collect any urine passed during the subsequent 8 hours in the collection bottle provided, rising exactly 8 hours after retiring, and emptying their bladder on rising into the bottle provided. They were also instructed to avoid alcohol on the day that they performed the debrisoquine test, and not to eat anything for 2 hours prior to taking the debrisoquine. In addition, all subjects who had a history of liver disease were excluded. Two 30-ml aliquots of the urine samples from each subject were made and frozen at -20^0C for later analysis; the concentrations of debrisoquine and 4-hydroxydebrisoquine in one of these were determined by flame-ionisation gas chromatography with minor modifications of the

Table 2.1 Experimental reagents

Reagent	Composition	Comments
Nonidet P40[R]	An octylphenol-ethylene oxide condensate containing an average of 9 moles of ethylene oxide per mole of phenol	
Proteinase K	20 mg/ml stock solution, use in reaction at 50 µg/ml	Store stock solution at –20^{0}C, digest at 37-56^{0}C
Proteinase K buffer	0.01 M Tris (pH 7.8), 0.005 M EDTA, 0.5% SDS	
10% Sodium dodecyl sulphate (SDS)	Dissolve 100 g SDS in 900 ml H_2O. Heat to 68^{0}C to assist dissolution. Adjust the pH to 7.2 by adding a few drops of concentrated HCl. Adjust the volume to 1 l with H_20.	A mask should be worn when weighing SDS and the weighing area and balance should be wiped down after use, because the fine crystals of SDS disperse easily. There is no need to sterilise 10% SDS.
1 M Tris·Cl	Dissolve 121.1 g Tris base in 800 ml H_20. Adjust the pH to the desired value by adding concentrated HCl (for pH 7.4, use approximately 70 ml HCl, pH 7.6, use 60 ml HCl, pH 8.0, use 42 ml HCl).	The pH of Tris solutions is temperature-dependent and decreases approximately 0.03 pH units for each 1^{0}C increase in temperature. For example, a 0.05 M solution has pH values of 9.5, 8.9, and 8.6 at 5^{0}C, 25^{0}C, and 37^{0}C respectively.
Phenol:chloroform	Mix equal amounts of phenol and chloroform, and equilibriate by extracting several times with 0.1 M Tris·Cl (pH 7.6-8.0), or buy equilibriated mixture. Store under an equal volume of 0.01 M Tris·Cl (pH 7.6-8.0), at 4^{0}C in dark glass bottles.	Phenol is highly corrosive, so safety glasses must be used, and all manipulations conducted in a hood. Any areas of skin coming into contact with phenol should be rinsed with a large volume of water and washed with soap and water.
TE	10 mM Tris·Cl (pH 8.0) 1mM EDTA (pH 8.0)	
Reagent A (Nucleon II Kit)	10 mM Tris·Cl 320 mM sucrose 5 mM $MgCl_2$ 1% Triton X-100	Adjust to pH 8.0 using 1M NaOH /concentrated HCl, and autoclave.
Triton X-100	Polyoxyethylene-p-isooctylphenol, a nonionic detergent	Store dark, at RT, can be used to substitute for Nonidet P-40 in many applications
Reagent B (Nucleon II Kit)	400 mM Tris·Cl 60 mM EDTA 150 mM NaCl 1% SDS	Adjust to pH 8.0 using 1M NaOH, prior to adding the EDTA. Autoclave prior to adding the SDS.
5 x TBE	270 g Tris Base 137.5 g Borate, 14.8 g EDTA	Make up to 5 litres with distilled deionised H_20

Table 2.1 Experimental reagents (continued)

PCR-grade H_20	Distilled deionised water, filtered through 0.2 μm filters	Or: water filtered using a MilliQ-Plus system
Ethidium bromide (10 mg/ml)	Dissolve 1 g ethidium bromide in 100 ml H_20 for several hours on magnetic stirrer (or buy solution at this concentration).	Store at RT in dark bottle. Ethidium bromide is a powerful mutagen, and appropriate precautions should be taken.
LB medium (1litre)	10 g Bacto-tryptone 5 g Bacto-yeast extract, 10 g NaCl	Make up to 1 litre with distilled deionised H_2O, then autoclave.
LB agar (350 ml)	1.5 g Bacto-tryptone 1.75 g Bacto-yeast extract 3.15 g NaCl, 5.25 g Bacto-agar	Make up to 350 ml with distilled deionised H_2O, then autoclave for 60 min.
6 x Loading buffer II	0.25% bromophenol blue 0.25% xylene cyanol FF 15% Ficoll (Type 44, Pharmacia)	Store at room temperature
6 x Loading buffer III	0.25% bromphenol blue 0.25% xylene cyanol FF 30% glycerol	Store at 4^0C
3M Sodium acetate	Dissolve 408.1 g sodium acetate·$3H_20$. Adjust the pH with glacial acetic acid, then adjust the volume to 1 l with H_20.	Dispense into aliquots and sterilise by autoclaving.
TEMED	N,N,N',N'-tetramethylethylenediamine	TEMED is a catalyst for the polymerisation of acrylamide when used with ammonium persulphate. Store well-sealed, dry, and dark, at RT.
Sequencing loading buffer	5:1 deionised formamide to 25 mM EDTA (pH 8.0), with blue dextran (50 mg/ml)	Store at -20^0C
IPTG	Isopropylthio-β-D-galactoside (MW 238.8 g). Dissolve 2 g IPTG in 8 ml distilled H_20. Adjust the volume to 10 ml with distilled H_20, and sterilise by filtration through a 0.2 μm filter.	Dispense the solution into 1 ml aliquots, and store at -20^0C.
X-gal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside. Dissolve in dimethylformamide to make a 20 mg/ml stock solution in a glass or polypropylene tube.	Wrap tube in foil and store at -20^0C. (Not necessary to sterilise by filtration.)
SOC medium	2% Tryptone, 0.5% Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM glucose (dextrose)	Store at -20^0C

method of Lennard *et al.* (1977), i.e. boiling for 15 min instead of incubating for 16 hours, and the use of a capillary column instead of a packed column. This assay was performed by M. Patel (St Bartholomew's Hospital); the second sample was stored for second analysis should this be required.

The debrisoquine metabolic ratio ($MR_{debrisoquine}$) was defined as the ratio of the concentrations of debrisoquine to 4-hydroxydebrisoquine in the urine sample. The limit of detection was 1 μg/ml, and subjects with no detectable metabolite were also classed as PMs. The intra- and interassay coefficients of variation (CV) for measurement of debrisoquine spiking the urine with 4 μg/ml, were 7.21% and 7.33% respectively. The corresponding values for 4-hydroxydebrisoquine were 7.97% and 9.98% respectively. Each assay was run with a control of known CYP2D6 intermediate metaboliser status ($MR_{debrisoquine}$ 4-6).

2.2.1.2 Dextromethorphan phenotyping

Dextromethorphan phenotyping, using a 30 mg dose of dextromethorphan and an 8-hour overnight urine collection was performed similarly. The concentrations of dextromethorphan and its *O*-demethylated metabolite, dextrorphan, in a 30 ml aliquot were determined by high performance liquid chromatography (Chen *et al.*, 1990), with the analysis being performed by collaborators at the Centre Hospitalier Specialisé. The dextromethorphan metabobolic ratio (MR) was defined as the ratio of the urinary concentrations of dextromethorphan to dextrorphan.

2.2.2 Isolation of DNA

2.2.2.1 DNA extraction from peripheral leucocytes

Blood (5-9 ml) was collected in potassium EDTA tubes and stored at -70^0C until use. Two methods of DNA extraction were used: phenol/chloroform (with proteinase K), and the Nucleon BACC3 kit for genomic DNA extraction (Tepnel Life Sciences PLC, Manchester, UK). The former was used for the samples collected earlier in this study, and the latter for the later samples, as it is a quicker method, more easily adaptable for the processing of considerable numbers of samples. The composition of all solutions used is given in Table 2.1.

Phenol/chloroform extraction of genomic DNA

Blood samples were thawed at room temperature, and decanted into 50 ml Falcon tubes. Distilled deionised H_2O at 4^0C was added to 40 ml, and the tubes placed on ice. Cells were then pelleted by centrifugation at 2,000 g for 10 min, resuspended in 25 ml 0.1 % Nonidet P40, and pelleted for a second time by centrifugation at 3,250 rpm for 10 min. This pellet was resuspended in 10 ml Proteinase K buffer. To this was added 25 µl proteinase K at 20 mg/ml (for protein digestion), then 0.5 ml 10% SDS (to lyse the nuclear membranes), the latter slowly, drop by drop, swirling the contents of the tube, until a viscous solution resulted. This was then incubated overnight at 37^0C. Tubes were then placed on ice again, 5 ml phenol at pH 7.8 (equilibriated with Tris.HCl pH 8.0) was added, the tubes were placed on a rotary shaker for 5 minutes, then centrifuged at 2,000 *g* for 5 minutes. The upper (aqueous) layer was then removed, to this was added 5 ml phenol at pH 7.8 and 5 ml chloroform, and the shaking and centrifugation steps were repeated as above. The aqueous layer was removed a

second time, and, either a phenol: chloroform extraction, or only chloroform extraction (5 ml) was repeated. The DNA was then precipitated using 2.5 times the volume of absolute ethanol at –20^{0}C, recovered using a flamed Pasteur pipette, air dried, and rinsed in 1 ml 70% ethanol at 4^{0}C. Depending on the yield, DNA was then resuspended in 50-200 µl of TE, left overnight at 4^{0}C to dissolve, and then stored at –20^{0}C prior to quantification.

Nucleon BACC3 kit extraction of genomic DNA

Blood was thawed as above, decanted into 15 ml Falcon tubes, and 14 ml Reagent A was added. Tubes were handshaken for 2 minutes, then placed on a rotary shaker for 5 minutes, then centrifuged at 2, 800 *g* for 8 minutes (to lyse the red cells and then pellet all cells). To the pellet was added 1 ml Reagent B, and this was vortexed vigorously until the pellet was completely dispersed (to lyse the leucocytes). To this was added 250 µl of 5M sodium perchlorate, the mixture was swirled and placed on a rotary shaker for 10 minutes, and then in a water bath at 63^{0}C for 25 minutes (effecting deproteinisation). To extract the DNA, 1ml of chloroform at –20^{0}C was added, the mixture swirled, and centrifuged at 2,000 *g* for 2 minutes. In order to facilitate separation of the upper DNA-containing aqueous phase from the lower organic phase, 200 µl of Nucleon II resin (a silica-based suspension) was added, and the tubes spun again at 2,000 *g* for 2 minutes. DNA was then precipitated by adding the upper aqueous layer to 2.5 ml absolute ethanol at –20^{0}C, and recovered, washed, dissolved, and stored as above.

Quantification of DNA

The concentration of the DNA in TE solution was measured using a UV spectrophotometer. The absorbance at 260 nm allows quantification of DNA, using the following equation:

$$[\text{DNA}] \text{ in } \mu g/ml = OD_{260} \times X \times \text{dilution factor}$$

where X is 50 for double-stranded DNA, and the dilution factor is that used for diluting the samples for reading in the spectrophotometer cuvette. A dilution factor of 20 was used (5 µl of sample diluted in 100 µl TE), with the result that the above equation simplifies to:

$$[\text{DNA}] \text{ in } \mu g/ml = OD_{260} \times 1000$$

$$\textit{i.e.} \quad [\text{DNA}] \text{ in } \mu g/\mu l = OD_{260}$$

In addition, the UV spectrophotometer readings were used to estimate the purity of the sample. The OD_{280} is a measure of the protein content, a ratio of the OD_{260} to OD_{280} of at least 1.8 indicating DNA of a high degree of purity; ratios of 1.6 and above were regarded as acceptable. After quantification, aliquots of the DNA samples at 100 ng/µl (in sterile TE) were made.

2.2.2.2 Isolation of DNA from agarose gels

The QIAEX II agarose gel extraction kit was used (designed for the extraction of 40 bp to 50 kb DNA fragments from 0.3-2.0% agarose gels). The DNA band of interest was excised from the gel using a clean scalpel and weighed in a 1.5 ml eppendorf tube. To

this was added 3 volumes of Buffer QX1 (containing a high concentration of chaotropic salt to disrupt the hydrogen bonds between the sugars in the agarose), then 10 µl of QIAEX II suspsension (containing glass powder to adsorb the DNA), then the eppendorf was inverted to mix contents, and incubated at 50^{0}C for at least 10 minute, with mixing of contents by hand every 2 minutes in order to keep the QIAEX II in suspension. The adsorption of DNA to QIAEX II is only efficient at pH $\subseteq$ 7.5, so once the gel had dissolved, the pH was checked (using pH paper), and if the pH was >7.5, 10 µl of 3M sodium acetate (pH 5.0) was added and the mixture incubated at room temperature for a further 10 minutes. The sample was then centrifuged at 10,000 *g* for 30 s, the pellet resuspended in 500 µl of buffer QX1 by vortexing, and then recentrifuged (to remove residual agarose contaminants). The pellet was then resuspended in 500 µl of Buffer PE by vortexing, and recentrifuged, with this step being repeated (to remove residual salt contaminants) At these last 3 stages, it is important to remove all traces of supernatant. The pellet was then air-dried at room temperature until it became white (usually approximately 15 minutes), following which the DNA was eluted by the addition of 20 µl of 10 mM Tris.HCl pH 8.5, resuspending the pellet by vortexing, incubating for 5-10 minutes at room temperature, and retrieving the supernatant after centrifugation.

2.2.2.3 Isolation of BAC DNA

The BAC clones were stored in *E. coli* cells at –70^{0}C, and grown up by seeding into conical flasks containing 250 ml of LB medium with chloramphenicol at 12.5 µg/ml (the latter to increase yield in the presence of the ColE1 replication origin), and incubating overnight in a shaking incubator at 37^{0}C. The bacteria were harvested by centrifugation at 2,400 *g* for 10 min, and the BAC DNA extracted by use of the

QIAGEN Plasmid Maxi kit (Qiagen Ltd., Crawley, UK), see Table 2.2 for composition of reagents.

The bacterial pellets were first resuspended in 10 ml Buffer P1 (in plastic centrifuge tubes), then 10 ml Buffer P2 was added, and the tubes inverted 10 times to mix contents, resulting in a viscous solution, which was incubated at RT for 5 min. To this was added 10 ml chilled Buffer P3, followed by immediate but gentle mixing of the tube contents by inversion 10 times, and incubation on ice for 20 min. Tubes were then centrifuged at at least 20, 000 x *g* for 30 min at 4^0C, and whilst this was being conducted, Qiagen-tip 500s were equilibriated by applying 10 ml buffer QBT, and allowing the column to empty by gravity-assisted flow. The supernatant was then filtered (filters supplied in syringes in the kit), allowing the filtrate to pass into the Qiagen tips, entering the resin by gravity flow. The Qiagen tips were then washed twice with 30 ml Buffer QC (this step removes contaminants), again allowing the buffer to pass through the tip by gravity flow. The DNA was then eluted with 15 ml Buffer QF into glass centrifuge tubes, and precipitated with 0.7 volumes isopropanol at RT by centrifugation at at least 15,000 x *g* for 30 min at 4^0C. The DNA pellet was then washed in 1 ml of 70% ethanol, then recentrifuged, this step being repeated up to 3 times, especially if not all of the pellet was recovered from the glass centrifuge tube initially. The BAC DNA was then dissolved in an appropriate volume of TE, overnight at 4^0C, and concentration estimated by running 1 µl on a 1% agarose gel together with standard DNA of known concentration.

2.2.2.4 Isolation of plasmid DNA

Single colonies containing plasmid DNA were picked from the sample plates, seeded in

Table 2.2 Experimental Materials - Qiagen Plasmid Purification Buffers

Reagent	Composition	Comments
Buffer P1 (resuspension buffer)	50 mM Tris.Cl, pH 8.0 10 mM EDTA 100 μg/ml RNase A	Use Tris base, adjust pH to 8.0 with HCl, store at 4^0C after the addition of the RNase A
Buffer P2 (lysis buffer)	200 mM NaOH, 1% SDS	Store at RT
Buffer P3 (neutralisation buffer)	3.0 M potassium acetate, pH 5.5	Store at RT, or 4^0C
Buffer QBT (equilibriation buffer)	750 mM NaCl 50 mM MOPS, pH 7.0 15 % isopropanol 0.15% Triton X-100	Store at RT, sterilise by filtration
Buffer QC (wash buffer)	1 M NaCl 50 mM MOPS, pH 7.0, 15% isopropanol	Store at RT, sterilise by filtration
Buffer QF (elution buffer)	1.6 M NaCl 50 mM Tris.Cl, pH 8.5 15% isopropanol	Store at RT, sterilise by filtration
Buffer QN (elution buffer)	1.6 M NaCl 50 mM MOPS, pH 7.0 15 % isopropanol	Store at RT, sterilise by filtration
STE	100 mM NaCl 10 mM Tris-Cl, pH 8.0 1 mM EDTA	Store at RT

to 5ml Circle Grow (BIO 101, Inc, Vista, CA, USA) medium with ampicillin at 50 ng/ml (for plasmids containing the ampicillin resistance gene, so that plasmids could be selected for ampicillin resistance), and placed in a 37^0C shaking incubator at 225 rpm overnight. The following day, 100 ml of Circle Grow medium with 50 ng/ml ampicillin was seeded with 500 μl of the 5 ml culture, and placed in the 37^0C shaking incubator again at 225 rpm for 16 hours. The next day, plasmid was harvested from the culture using QIAGEN-tips 500 (Maxi), following the same plasmid purification protocol as above, except that I found that in order to produce high quality plasmid, it

was necessary to vortex the pellet briefly in the first step, to perform a second centrifugation step (recentrifuging at 20,000 x *g* for 15 min at 4^0C after the first centrifugation at 20,000 x *g*), to perform a second wash with 70% ethanol at the end, and to ensure that the pellet was dry (translucent) before redissolving in TE pH 7.5 (100 µl).

2.2.3 PCR techniques

2.2.3.1 Standard length PCR

Primer-mediated enzymatic amplification of DNA was first described by Saiki *et al.* (1985), and was named the polymerase chain reaction by Mullis *et al.* (1986, 1987). It is a technique for amplifying *in vitro* a segment of DNA that lies between two regions of known sequence, commencing with primers (oligonucleotides) of sequence complementary to opposite strands of the known sequence, at opposite ends of the target region to be amplified. The DNA is amplified exponentially by a series of repeated cycles of denaturation of template, primer annealing, and extension of sequence beginning with the annealed primers by a DNA polymerase. The extension product of one cycle of the PCR reaction serves as the template in the next cycle, causing an exponential accumulation of the product. It is theoretically possible to achieve a 10^7 fold amplification in 30 rounds of thermal cycling. The efficiency of a PCR amplification can be calculated using the following formula:

$$N = N_0 (1+eff)^n$$

Where N = number of end products, N_0 = number of starting targets (1 μg of human genomic DNA =1.4-3 x 10^5 copies of a single-copy gene), n = number of cycles, and eff = mean efficiency of a cycle. For example, with an efficiency of 85%, 20 cycles will give 220,000-fold amplification.

The DNA polymerase used for standard length PCR (<5 kb) was *Taq*, a thermostable DNA polymerase purified from the thermophilic bacterium, *Thermus aquaticus*, and produced by the expression of a modified form of the *Taq* DNA polymerase gene cloned in *E coli* (Lawyer *et al.*, 1989). This is a 94 kDa protein that has a $t_{1/2}$ of approximately 40 minutes at 95^0C, possesses 5'-3' exonuclease activity, but lacks 3'-5' exonuclease activity, and extends at a rate of 2-4 kb per minute at 72^0C. The fidelity of *Taq* is less than or equal to 1 in 10-50,000 nucleotides (Eckert & Kunkel, 1991), and is affected by reaction conditions (*e.g.* high magnesium concentrations reduce the fidelity of *Taq*).

The primer sequences were designed either by hand, using certain guidelines (*e.g.* a "GC clamp" at the 3' end, *i.e.* a G or a C at the 3' end of the sequence, may increase specificity of annealing), or using the programme PRIMER (Lincoln *et al.*, 1991), and all primer sequences can be found within the relevant chapters of this thesis. Primers that were designed by hand were tested for suitability using web-based tools for identification of likelihood of secondary structure formation, *i.e.* inter-primer dimers/oligomers or intra-primer hairpins (*e.g.* that available via Operon Technologies, Inc. on the website http://operon.com). The T_m (melting temperature) of each primer was calculated using the following formula for primers of at least 10 bases:

$$T_m = 81.5 + 16.6\,(\log[\text{molar J+}]) + 41(\#G + \#C)/\text{primer length} - 675/\text{primer length}$$

Where the molar J+ is the concentration of monovalent cations and is 0.05 M for PCR (see below), and #G is sum of the number of Gs, and #C the sum of the number of Cs in the primer. For primers of less than 10 bases, the following formula is adequate:

$$T_m = (\#G + \#C) \times 4 + (\#A + \#T) \times 2$$

Primer pairs were designed such that their T_ms were similar, and chosen such that in general the %GC (*i.e.* 100 x (#G +#C/primer length)) was close to 50%, or if the region to be amplified was such that primers with less than 50% GC had to be used, then the length of the primer was increased. Sources for the primers included Oswel DNA Service, GibcoBRL, Operon Technologies, Inc., and Genset. Primers in excess of 30 base pairs were HPLC-purified. The annealing temperature (see below) was initially set at 5^0C below the mean T_m of the primer pair.

Various thermal cyclers were used for the PCRs (e.g. Perkin Elmer Thermal Cycler, Perkin Elmer 2400, Biometra TRIO-Thermoblock, MJ Research DNA Engine Tetrad), and the cycling conditions varied slightly depending on the cycler used, and again, specific conditions (annealing temperatures, etc.) are given in the relevant chapters of the thesis. In general, the cycling conditions included an initial denaturation step of 93^0-95^0C for up to 3 min, in order to denature genomic template, followed by 25-35 cycles of denaturation at 93^0C for 30s to 1 min, annealing at 50-65^0C depending on the primer T_ms for 10 s – 1 min, and elongation at 72^0C for 1-3 minutes. Slightly lower temperatures and slightly shorter time periods were chosen for the later model cyclers (employing a more rapid ramping system). The length of the elongation step was

chosen according to the length of the amplicon expected, at 1 minutes per kb. Finally, a terminal elongation step of 72^{0}C for 7 minutes was performed, in order to increase the number of full-length products, since in the final rounds of amplification the amount of enzyme may become limiting and lead to the production of partially elongated products. The reaction was then cooled to 4^{0}C.

The primers were used together with 50-100 ng genomic DNA in a total reaction volume of 25-100 µl, containing 10 mM Tris.HCl pH 8.3, 50 mM KCl, 0.001% (w/v) gelatin, 1-3 mM $MgCl_2$, 0.2 mM each dNTP, 0.2-0.5 µM each primer, 1-2.5 U *Taq*, and made up to the total reaction volume with PCR-grade water (Table 2.1). The mixture of the Tris.HCl, KCl, and gelatin was supplied as a 10 x concentrate ("PCR buffer") together with the *Taq* supplied by manufacturers (a variey of manufacturers were used during the course of the work for this thesis, including Applied Biosystems (Perkin-Elmer) and Bioline, most of the work being conducted using *Ampli Taq* supplied by Applied Biosystems). A negative control (PCR-grade water of equal volume to that used of the genomic DNA sample) was included for each set of PCR reactions, in order to ensure that no contamination had entered the reactions. The reaction conditions for each primer pair were initially set at 1.5 mM $MgCl_2$ and 0.2 µM each primer, and if yield or specificity was inadequate with this, then optimisation of the reaction was conducted, according to the following principles:

1 Increasing the annealing temperature closer to the T_m of the primers enhances specificity of annealing, and reducing the length of the time at the annealing temperature may reduce the likelihood of extension occurring from a misprimed event (*i.e.* where the primer has annealed to a sequence that it is not exactly complementary to).

2 Reducing the number of cycles may also increase the specificity. In general, 25 cycles should be sufficient, and the lowest cycle number that results in an adequate amount of PCR product for subsequent applications should be used.

3 A "hot start," *i.e.* not allowing *Taq* to have contact with the full complement of reagents until the reaction temperature is at least 80^0C, increases the specificity of the reaction, and can be achieved by techniques such as the use of AmpliWax™ (Perkin-Elmer, UK, see below). Similarly, a "pseudohotstart," *i.e.* heating the thermal cycler to 90^0C before adding the reagents, may also achieve this.

4 Increasing the $MgCl_2$ concentration may increase the yield, but will tend to reduce the specificity. The magnesium concentration affects primer annealing, strand dissociation temperatures of the template and PCR product, formation of primer-dimer artefacts, and *Taq* activity and fidelity.

5 Reducing the dNTP concentration reduces the likelihood of extending misincorporated nucleotides (Innis *et al.*, 1988). The use of nucleotide stocks that have been subjected to multiple freeze-thaw cycles should be avoided.

6 Increasing the primer concentrations may promote mispriming and increase the probability of generating primer-dimers.

7 Increasing the amount of *Taq* above 2.5 U per reaction may lead to the accumulation of non-specific products. However, an inadequate amount of *Taq* (*e.g.* <1 U) may lead to the plateau phase of the PCR reaction (at which amplification of target sequences is no longer exponential) being reached before the end of the PCR programme, especially for cycle numbers greater than 25. As the cycle number increases, there may eventually be less than one molecule of *Taq* per primer-template complex, which will contribute to the plateau

phenomenon, yielding less than full length product, which is then unable to serve as intact template for further rounds of amplification. The use of an autosegment extension as part of the cycling programme (where the denaturation time is extended in later cycles) can delay the plateau effect.

8 The use of cosolvents (up to 15% glycerol, 5-10% DMSO, or 0.05-0.5% $Tween_R$ 20) may enhance the denaturation GC-rich targets, and improve the yield, especially of relatively long targets (see below).

Usually optimisation by trying alternative annealing temperatures (*e.g.* 2^0C-10^0C above and below the mean T_m of the primers) and alternative $MgCl_2$ concentrations (within the ranges of 1-3 mM) was sufficient to produce pure product of adequate yield.

2.2.3.2 Long PCR

Although it is possible to produce amplicons of 5-15 kb with *Taq*, the reported yields are low, so where the target length of DNA was at least 3 kb, the technique known as "long PCR" was used. This refers to a series of modifications of standard PCR, first described by Cheng *et al.* (1994) and Barnes (1994), including:

2 Two-temperature thermal cycling (one temperature for denaturation, and another temperature for both primer annealing and strand extension), with a denaturation step at no higher than 94^0C in order to minimise DNA damage, and annealing/extension at 68^0C. The high annealing temperature minimises the formation of products from secondary priming sites. Primers that are 20-23 oligomers of 52-60% G+C content in general are successful with a 68^0C annealing and extension temperature. An annealing/extension time of 5 min is sufficient for products of up to approximately 9 kb, with times of up to 30 min

being necessary for targets of up to 40 kb, and including an autosegment extension of 15-20 s per cycle for the later cycles. A hot-start method also minimises undesirable primer interactions, and can either be achieved through the use of a "pseudohotstart" (as described above), or through the use of Ampliwax.™ Ampliwax beads are specially formulated wax beads of an appropriate size that melt at 80^0C; in the use of Ampliwax, a lower layer of long PCR reagents (long PCR buffer, dNTPs, primers and $Mg(OAc)_2$) is added to the tubes first, followed by a wax bead, the wax is then melted by placing the tubes in a thermal cycler for 5 minutes at 80^0C and then cooled to room temperature, following which the upper layer of long PCR reagents are added (more long PCR buffer, long PCR enzyme, and target DNA). The reactions are then placed in the cycler, and during the first cycle of amplification, the wax melts, allowing the 2 layers to mix, with the result that the long PCR enzyme has the full conditions necessary for its function once the reaction is at at least 80^0C. The melted wax then serves as a vapour barrier for the reset of the amplification (for older cyclers that do not use a heated lid protocol).

3 The addition of a small amount of a thermostable DNA polymerase that has 3' to 5' exonuclease in addition to the primary polymerase greatly improves yield (Barnes, 1994). The 3'-5' exonuclease activity acts as a "proofreader," removing any mismatched nucleotides and permitting the predominant polymerase activity to complete the strand synthesis. One such mixture of enzymes is r*Tth* + Vent^R, marketed by Applied Biosystems (Perkin Elmer) in their GeneAmp XL PCR kit; another is *Taq* + *Pwo* marketed by Boehringer Mannheim in their Expand Long Template PCR kit, where Vent^R and *Pwo* are

the 2 proofreading enzymes. I used both of these kits, for different long PCR applications.

4 The addition of cosolvents as well as the proofreading enzyme greatly improves yield. Cosolvents may increase the thermal stability of the polymerase, and effectively lower melting and strand separation temperatures (2.5-3^{0}C per 10% glyerol), thus facilitating denaturation of the template and increasing the specificity of primer annealing. Cheng *et al.* (1994) demonstrated that for long PCR, the use of more than one cosolvent (*e.g.* DMSO with glycerol) was more effective than one, and the buffers in the GeneAmp XL and Expand Long Template PCR kits both contain more than one cosolvent (tricine, DMSO, and glycerol in the former in addition to potassium acetate, concentrations not given by manufacturer; and in the latter: 20 mM Tris.HCl pH 7.5, 100 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA, 0.5% (v/v) Tween$_R$ 20, 0.5% (v/v) Nonidet$_R$ P40, 50% glycerol (v/v), and 1.75 mM $MgCl_2$). The use of the Tricine buffer enhances the activity of r*Tth*, but Tris.HCl is better for Taq, while lower K^+ concentrations, and the use of KOAc rather than KCl also enhance the yield.

For my first experiments with long PCR, I used the GeneAmp XL PCR kit, and the control λ DNA and λ primers supplied with the kit. I was able to successfully generate a 20.8 kb amplicon, following the manufacturer's instructions. Specifically, the reaction conditions were: 0.01 μg λ DNA, 1 x XL Buffer, 0.2 mM dNTPs, 0.4 μM each primer, 1.1 mM $Mg(OAc)_2$, and 4U r*Tth* XL in a final total reaction volume of 100 μl having done the two-layer technique with AmpliWax as described above. The cycling conditions (in a Perkin Elmer DNA Thermal Cycler) were: 93^{0}C for 1 min; 16 cycles of 93^{0}C for 1 min, 68^{0}C for 10 min; 12 cycles of 93^{0}C for 1 min, 68^{0}C for 10 min with a

15 s increment per cycle; and a terminal elongation step of 72^0C at 10 min, followed by cooling to 4^0C.

I then applied this technique to the amplification of the intergenic segments between *CYP2D8P*, *CYP2D7P*, and *CYP2D6*, in order to generate a PCR that was capable of detecting extra copies of *CYP2D6* (*CYP2D6* gene amplification). For this purpose, sequence comparison of *CYP2D8P*, *CYP2D7P*, and *CYP2D6* was undertaken, and primers were designed from regions of complete homology in exons 9 and 1. The forward primer was designed from the forward sequence of exon 9 near its 3' end, sequence: 5'-CTTTGTGCTGTGCCCCGCTAG-3.' The reverse primer was designed from the reverse sequence of exon 1, near its 5' end, sequence 5'-GTCCACCAGGAGCAGGAAGAT-3'. Primers were used at concentrations of 0.2 μM, with 300 ng genomic DNA, and reaction conditions and cycling parameters were otherwise as for the amplification of the λ DNA. This produced a successful result, with the generation of 2 amplicons, representing the *CYP2D8P-CYP2D7P* and the *CYP2D7P-CYP2D6* intergenic segments. Figure 2.1 shows the 2D locus with intergenic products expected for different allelic variants. Optimisation of the reaction was then conducted, using different amounts of genomic template (Figure 2.2), which demonstrated that 400 ng of genomic DNA gave the best results with this protocol. I then performed this PCR on the 163 French subjects, which demonstrated that in one subject, of very low dextromethorphan MR (0.002), there was an extra band, corresponding to a *CYP2D6-CYP2D6L* intergenic segment (Figure 2.3), of length approximately 8.5 kb (Aitchison *et al.*, 1995a). I also performed restriction analysis of the long-PCR products (Figure 2.4). The 4.8 kb *2D8P-2D7* fragment was cleaved twice by *Xba* I, giving fragments of 0.7, 1.9, and 2.2 kb. Only 1 site was expected from

the map given in Kimura *et al.* (1989), but on checking the *Xba* I sites in the known sequence (Genbank accession number M33387) using the HGMP sequence analysis tools, the 2 sites were confirmed, with positions as shown in Figure 2.1. The 4.8 kb fragment was also cleaved once by *Eco*R I, as expected, giving fragments of 1.8 and 3.0 kb, while the 9.5 kb *2D7P-2D6* fragment was also cleaved only once by *Eco*R I, giving fragments of 7.9 and 1.6 kb. I also further verified the methodology of this technique through the testing of a positive control, kindly supplied by Dr Marja-Liisa Dahl (Karolinska Institute, Huddinge University Hospital, Sweden), which was previously described as having results on pulse field gel electrophoresis consistent with 13 copies of CYP2D6 (Johansson *et al.*, 1993). Figure 2.5 shows the results of my *CYP2D* intergenic long PCR on this subject, showing that the band corresponding to the *CYP2D6-CYP2D6L* intergenic segment has a very high intensity, consistent with multiple copies of this region, of the same length.

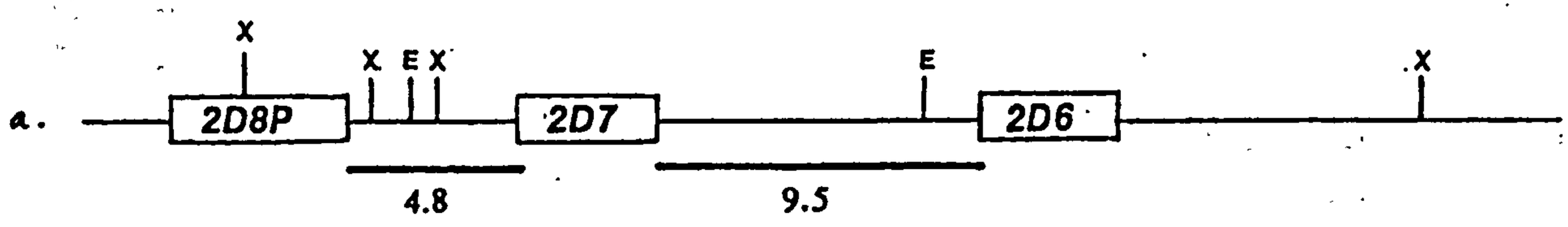


Figure 2.1 *CYP2D6* alleles, as identified by novel long-PCR technique. *Xba* I and *Eco* RI restriction sites are denoted by X and E respectively. Expected PCR amplicons (numbers giving length in kb) are shown in bold under the map, for wild-type allele (a), *CYP2D6*5* (b), and CYP2D6 gene duplication (c).

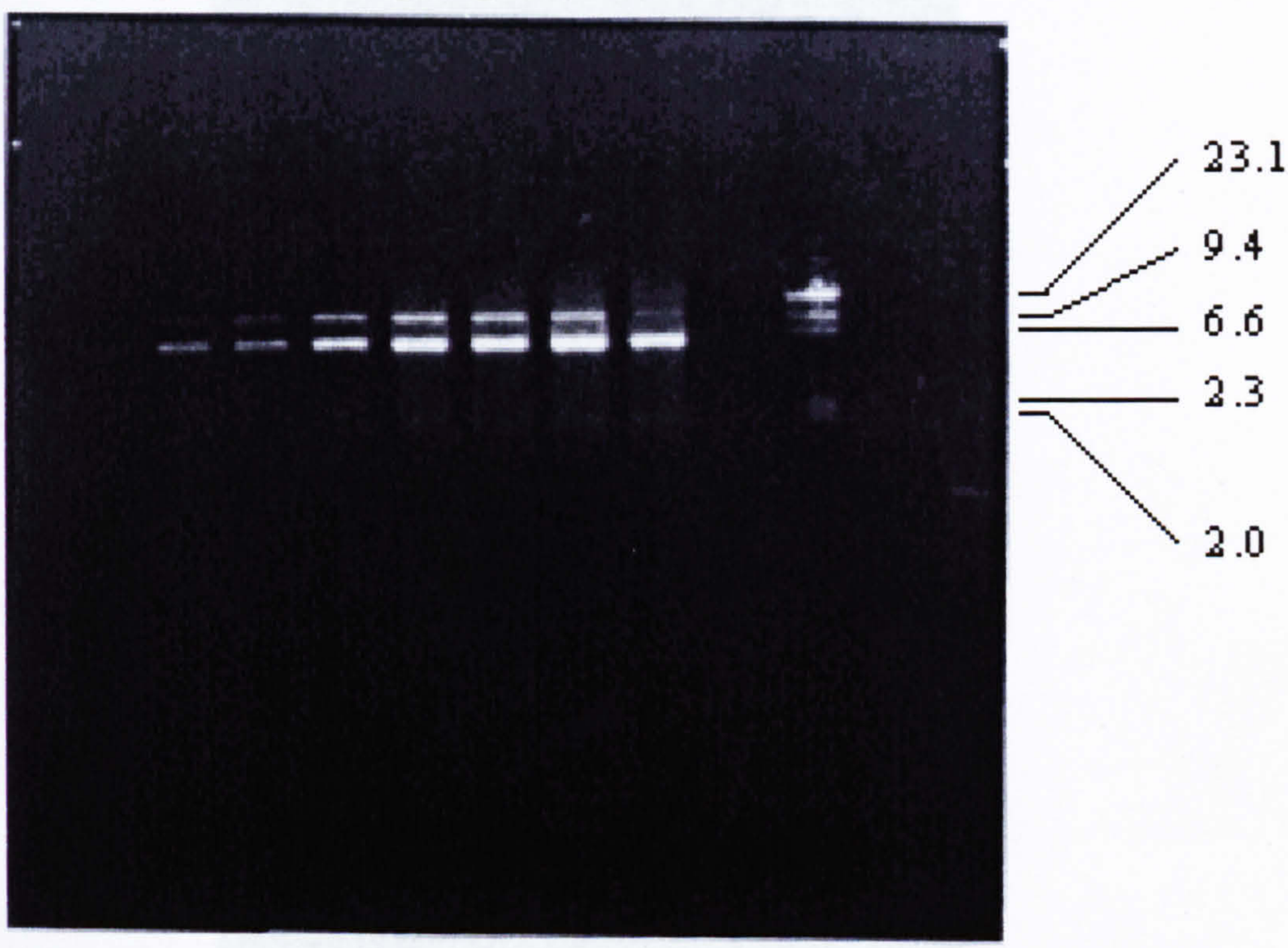


Figure 2.2 Optimisation of CYP2D intergenic long-PCR technique: the effect of increasing template concentration. Lanes 1-8 have 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, and 0.9 μg respectively (negative control in lane 9, λ DNA/*Hind* III ladder (kb) in lane 10).

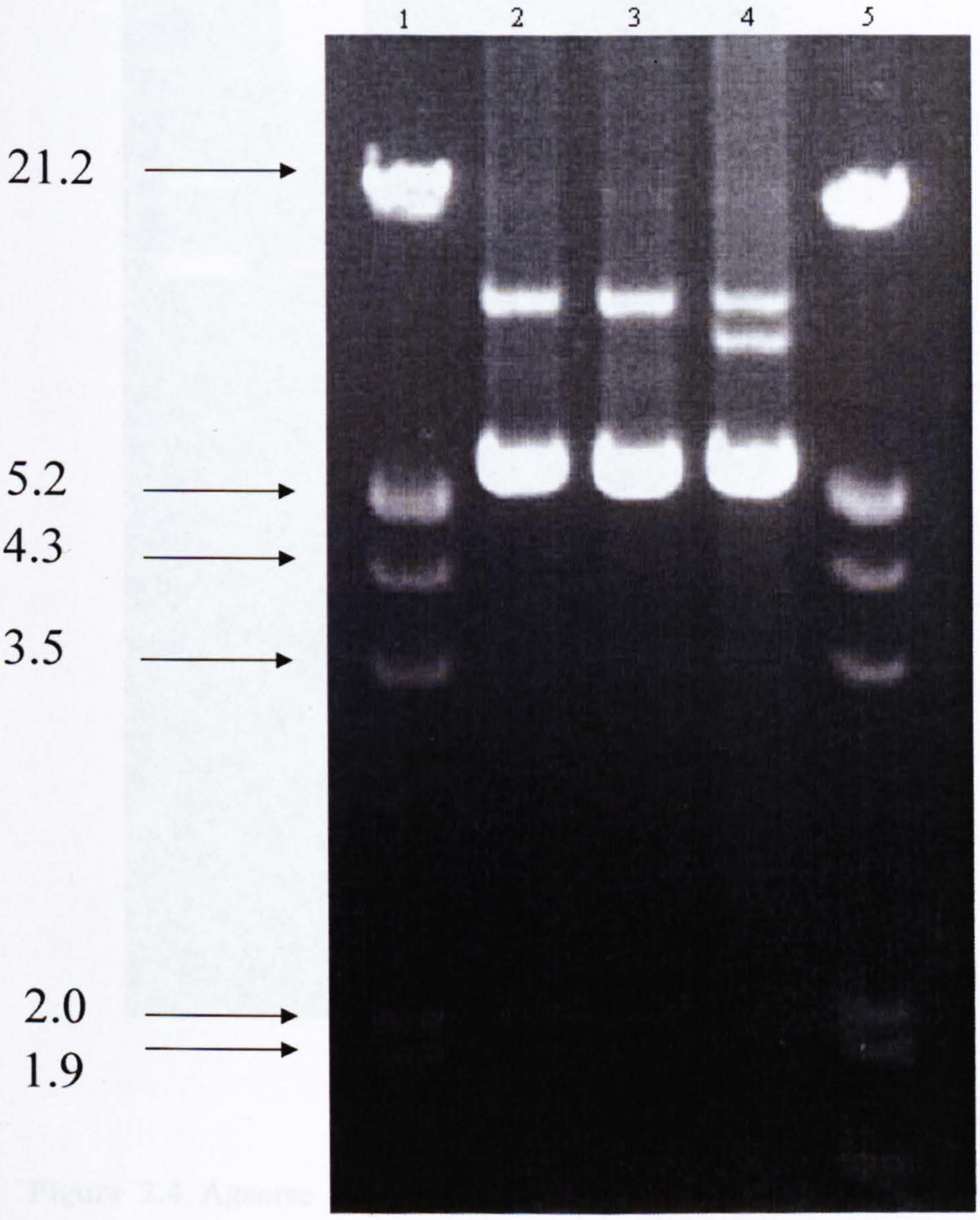


Figure 2.3 Agarose gel electrophoresis of products of novel long-PCR for the identification of *CYP2D6* gene amplification. Subjects of lanes 2 and 3 are extensive metabolisers; in lane 4 is the result for an ultrarapid metaboliser (dextromethorphan MR 0.002). In lanes 1 and 5 are a λ DNA/*Eco* RI + *Hind* III ladder (fragment lengths given).

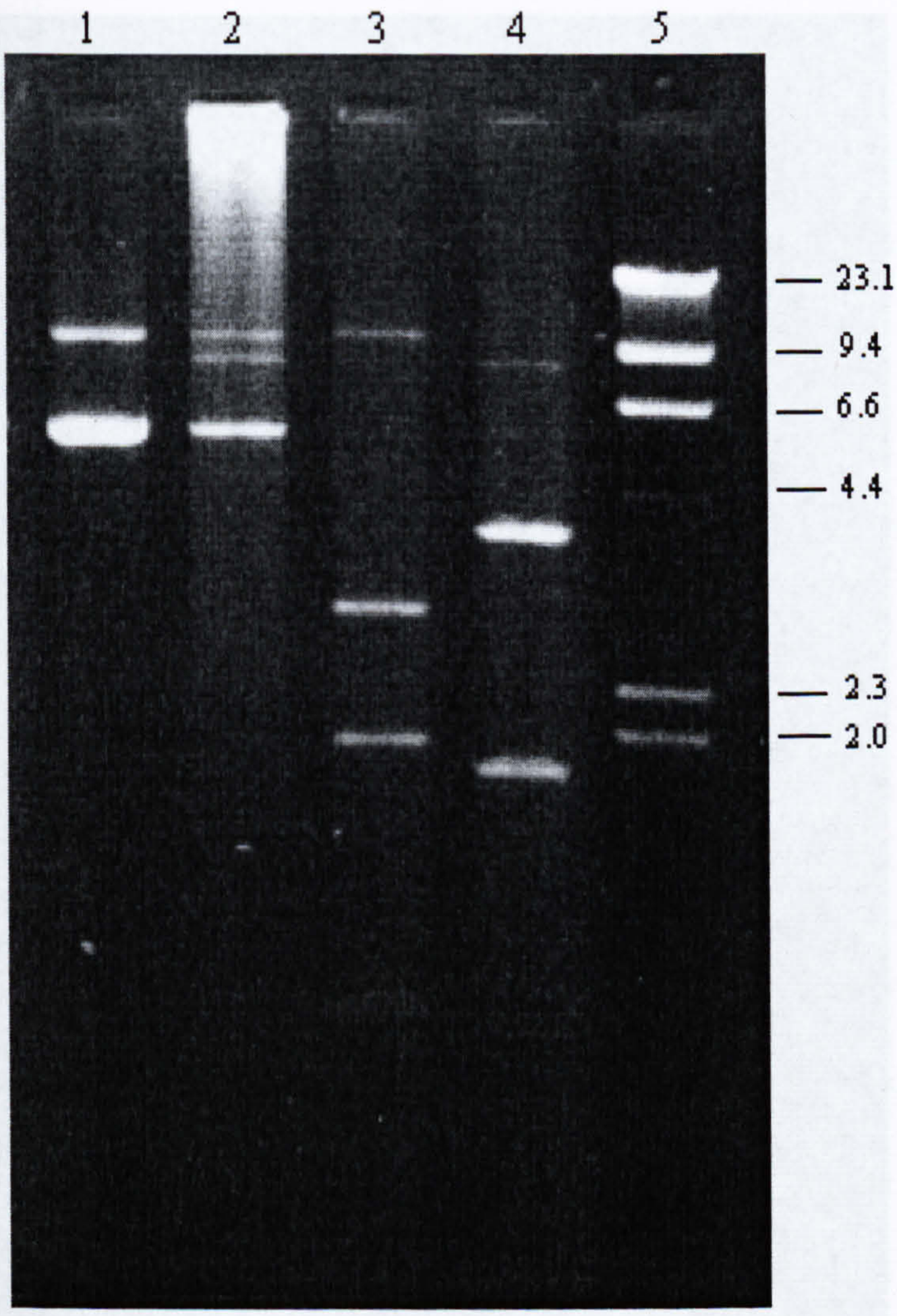


Figure 2.4 Agaorse gel electrophoresis of novel *CYP2D6* long-PCR products and restriction enzyme digests of products (0.6% agarose). Lane 1 shows wild-type banding (4.8 and 9.5 kb), lane 2 the extra band of 8.5 kb in subject with dextromethorphan MR of 0.002. Lane 3 shows *Xba* I digest of products in lane 1, and lane 4 *Eco* RI digest of lane 1 products. A λ/*Hind* III ladder (kb) is in lane 5.

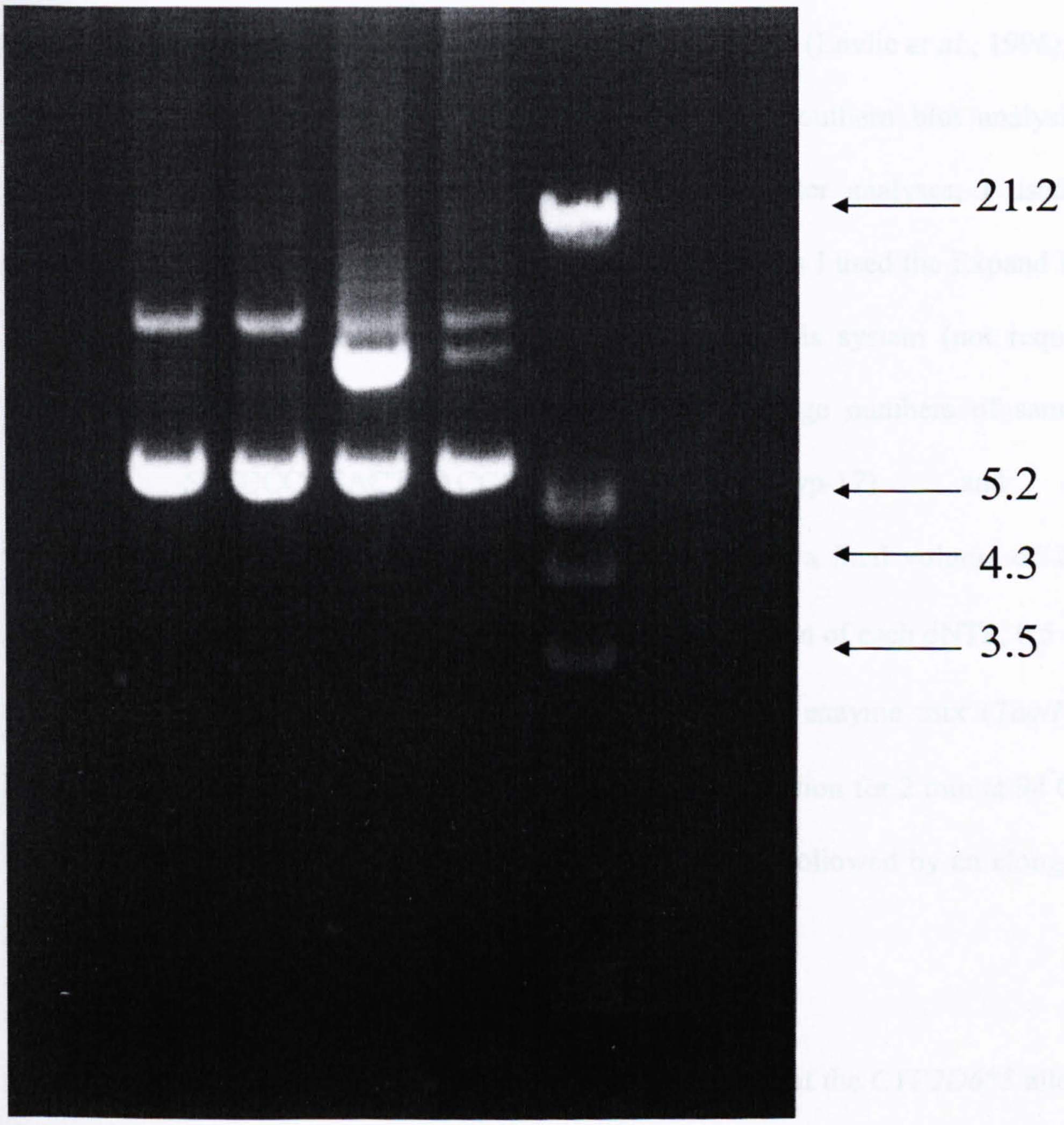


Figure 2.5 Agarose gel electrophoresis of products of novel *CYP2D6* long-PCR assay, showing increased intensity of 8.5 kb amplicon in subject known to have 13 copies of CYP2D6 (lane 3), as compared to extensive metabolisers (lanes 1 and 2) and the ultrarapid metaboliser with dextromethorphan MR of 0.002 (lane 4). In lane 5 is a λ DNA/*Eco* RI + *Hind* III ladder. DNA from subject with 13 copies of CYP2D6 kindly supplied by Marja-Liisa Dahl.

However, during the time of my PhD work, another group also published a long PCR method for the identification of CYP2D6 amplification events (Løvlie *et al.*, 1996), and as their work was more thorough than mine, including Southern blot analysis of subjects and sequencing of the intergenic product, for later analyses, I used the methodology of this group, with minor modifications. For this I used the Expand Long Template PCR System (Boehringer Mannheim, UK), as this system (not requiring AmpliWax) lends itself more easily to the analysis of large numbers of samples. Primers 5'-TCCCCCACTGACCCAACTCT-3' (cyp-17) and 5'-CACGTGCAGGGCACCTAGAT-3' (cyp-32) were used in a final volume of 25 µl including 2.5 µl Boehringer buffer 1, 4.5 µl of a 2 mM solution of each dNTP, 0.5 µl of each primer (10 µM solutions), and 0.25µl of Boehringer enzyme mix (*Taq/Pwo*). Cycling conditions were modified as follows: initial denaturation for 2 min at 94°C; 35 cycles of 93°C for 10 s, 60°C for 30 s and 68°C for 5 min; followed by an elongation step of 68°C for 7 min.

Long PCR methodology was also used for the identification of the *CYP2D6*5* allele as described by Steen *et al.* (1995), using primers 5'-ACCGGGCACCTGTACTCCTCA-3' and 5'-GCATGAGCTAAGGCACCCAGAC-3' and the GeneAmp XL PCR kit. PCR was performed using Ampliwax$_R$ beads to facilitate a hot start in a 100 µl reaction volume with 200 ng genomic DNA, 1xXL reaction buffer, 0.2 mM each dNTP, 0.3 µM each primer, 1.1 mM $Mg(OAc)_2$ and 2U of the r*Tth*/Vent$_R$ DNA polymerase mixture. Cycling conditions were: initial denaturation at 93^{0}C for 1min; 35 cycles at 93^{0}C for 1 min, 65^{0}C for 30 s, 68^{0}C for 5 min; and a final elongation at 72^{0}C for 10 min.

Duplications of non-functional alleles (e.g. *CYP2D6*4*) have been described, and

therefore, if cases were positive on both the *CYP2D6*4* and the duplication assays, they were further tested to determine whether the null or the wild type allele was duplicated as described by Sachse *et al.* (1997): a further duplication assay was performed with primers as described by Johansson *et al.* (1996), giving a 10kb amplicon in cases positive for a duplication, which was then subjected to a nested PCR followed by digestion with *Hph* I (Figure 2.6). The primers for the Johansson *et al.* assay were 5'-GCCACCATGGTGTCTTTGCTTTC-3' and 5'-ACCGGATTCCAGCTGGGAAATG-3', and I modified the conditions by performing the assay with the Expand Long Template PCR System, using a total reaction volume of 25 µl with 2.5 µl of Boehringer buffer 1, 4.5 µl of a 2mM solution of each dNTP, 0.5 µl of each primer (10 µM solutions), 0.37 µl of the *Taq*/*Pwo* enzyme mix, and 200 ng of genomic DNA. Cycling conditions were: initial elongation of 94^{0}C for 2 min; 10 cycles of 93^{0}C for 10 s, 60^{0}C for 30 s, 68^{0}C for 12 min; 20 cycles of 93^{0}C for 10 s, 60^{0}C for 30 s, and 68^{0}C for 12 min with a 15 s increment per cycle; and a terminal elongation step of 68^{0}C for 7 min. The product of this reaction was then diluted 1:5 and subjected to nested PCR using primers 5'-TCAACACAGCAGGTTCA-3' and 5'-CTGTGGTTTCACCCACC-3'. The reaction volume was 65 µl, containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.160 mM each dNTP, 0.1 µM each primer, and 0.2 U *AmpliTaq*. The cycling conditions were: initial elongation at 94^{0}C for 2 min, 25 cycles of 94^{0}C for 30 s, 58^{0}C for 10 s, 72^{0}C for 1 min, and a final elongation step of 72^{0}C for 7 min. The products were digested with *Hph* I and separated on a 3% agarose gel (see below).

2.2.4 Agarose gel electrophoresis

0.7-3% (w/v) agarose gels were prepared by adding the appropriate weight of agarose to the appropriate volume of 1x TBE (Table 2.1) buffer, and dissolved by heating in a

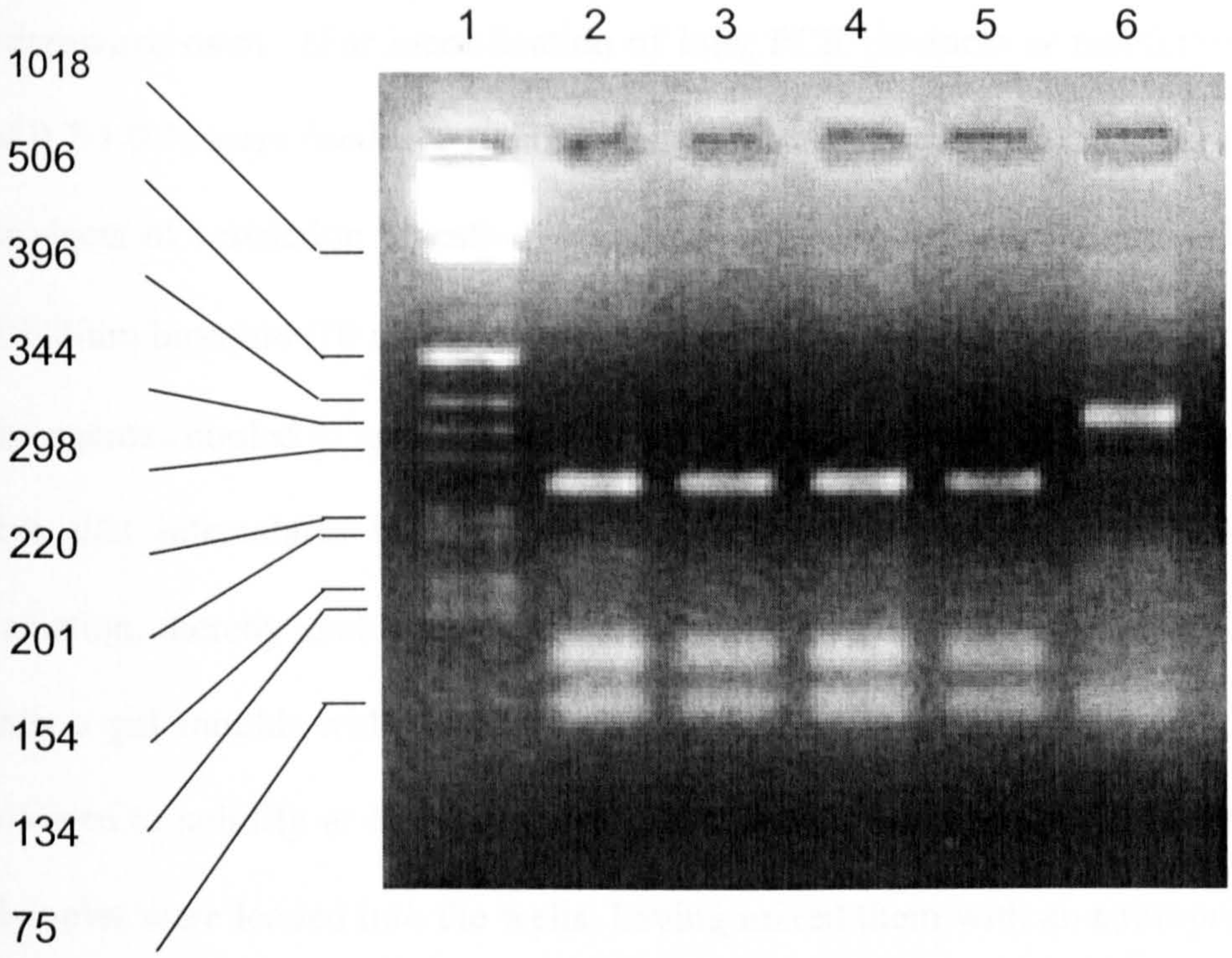


Figure 2.6 Agarose gel electrophoresis of *Hph* I digests for *CYP2D6*4x2* analysis. Subjects in lanes 2 to 5 are all positive for this variant, while the subject in lane 6 has a gene duplication that is not *CYP2D6*4x2* (is *CYP2D6 wt/wt* on the *CYP2D6*4* assay, and positive for a gene duplication event on the Løvlie *et al*., 1996 assay). In lane 1 is a 1 kb ladder (Life Techologies, Inc., bp).

microwave oven. (For identification of long PCR products or restriction digests, gels of 0.7-1.0 % were used; for standard length PCR products, 1% gels were used; and for products of restriction digests of standard length PCR products, 3% gels were used.) Ethidium bromide (10 mg/ml) was then added to a final concentration of 0.5 μg/ml, and the agarose cooled to approximately 70^{0}C on a magnetic stirrer. Ethidium bromide is a dye that intercalates between the base pairs of DNA, and fluoresces under UV radiation, thereby enabling visualisation of the DNA. The cooled agarose was poured into a gel mould, with casting combs to generate an appropriate number of wells, allowed to solidify at 4^{0}C, then placed in an electrophoresis tank containing 1 x TBE. Samples were loaded into the wells, having mixed them with an appropriate volume of 6 x loading buffer. For agarose gel electrophoresis of long PCR products, it was found that loading buffer II (with Ficoll) gave a better result. For most other applications, loading buffer III was used. An appropriate size marker (100 bp or 1kb ladder for standard length PCR or PCR-RFLP analysis, λ *Hind* III or λ *Hind* III/*Eco*R I for long PCR) was loaded in addition to the samples. The gel was electrophoresed at an appropriate voltage/cm (negatively charged DNA migrates towards the positive electrode) until the required separation had been achieved, and then photographed under UV light.

2.2.5 RFLP analysis

Restriction endonucleases (REs) are enzymes purified from bacteria that cut DNA at specific recognition sequences (often palindromic in form, *e.g.* GTTAAC, the sequence on one strand being the same as that on the other in the reverse direction). This property can be used in mapping (*e.g.* confirmation of the identity of long PCR products), or in identification of polymorphic sites in a sequence. Restriction fragment length

polymorphism (RFLP) refers to variations in lengths of DNA produced by digestion with a particular RE owing to the presence/absence of the RE site in that particular region of DNA from different individuals. PCR-RFLP analysis refers to the generation of a PCR product that contains a polymorphic site from a number of different individuals, followed by restriction digestion of the products, in order to identify which individuals carry the variant site. The polymorphic site is a single nucleotide polymorphism (SNP) within the RE recognition sequence so that the enzyme either cuts or fails to cut according to the presence/absence of the SNP. Examples of such PCR-RFLP analyses performed include the *CYP2D6*4* and *CYP1A2* $T_{-3591}G$ identification. If the SNP to be identified does not alter a restriction site, mutagenic primers can be designed, which introduce a restriction site overlapping the position of the sequence change. An example of such an analysis is the *CYP2D6*3* assay.

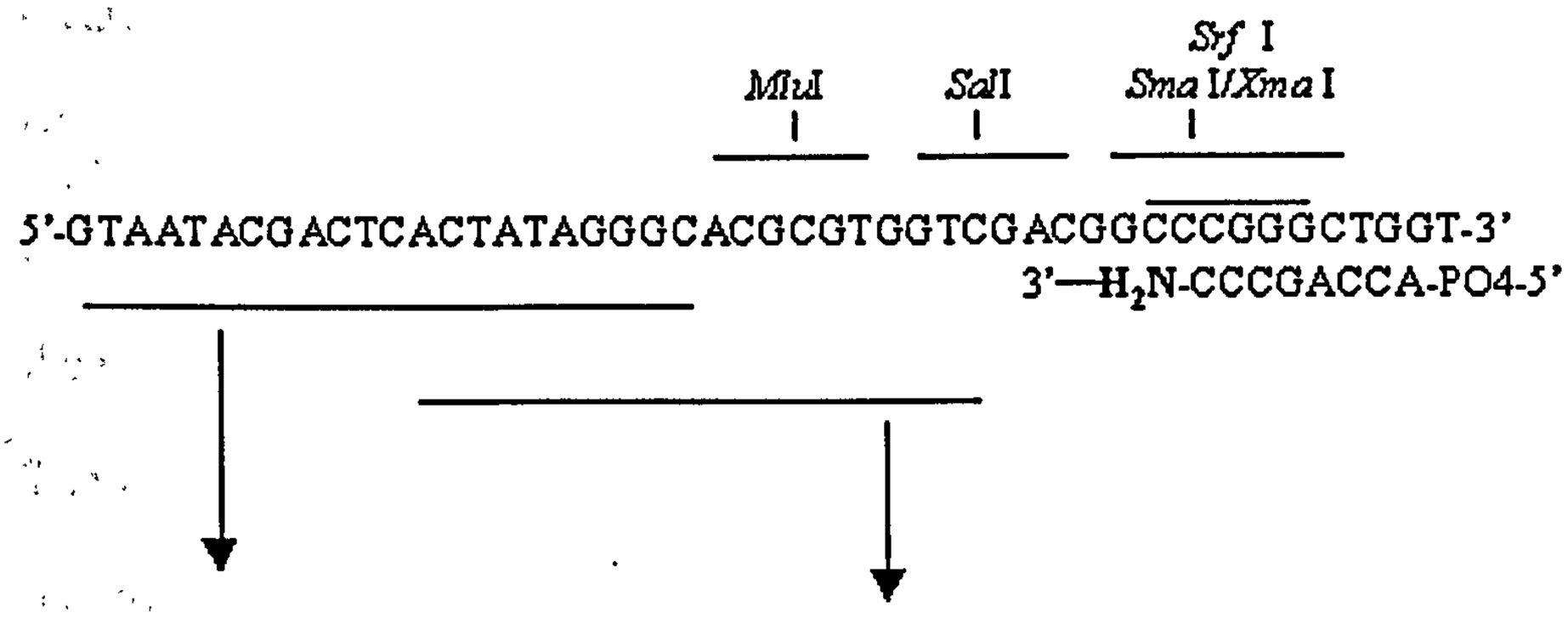
PCR products were incubated with the appropriate RE, the RE buffer as supplied by the manufacturer, and, if indicated by the manufacturer, BSA to a final concentration of 100 μg/ml. In general 5-10 U of RE were used for up to 20 μl of PCR product, but for the case of *Mbo* II digestion (identification of the $T_{-3591}G$ SNP), a series of assays determined that 1.5 U gave best results (for up to 20 μl PCR product). The incubation temperatures were as recommended by the manufacturer, and conducted in an incubator set at the appropriate temperature (e.g. 37^0C) or a Hybaid oven (for the higher temperatures, e.g. 60^0C for *Bst*N I).

2.2.6 Genome walking

"Genome walking" is a technique for peforming serial PCR of genomic segments from a region of known sequence into a region of unknown sequence. Primers were designed

for use with the GenomeWalker™ Kit (Clontech Laboratories, Inc., CA, USA) in order to generate genomic sequence further 5' than the 5' flanking sequence of *CYP1A2* that had been published to date (the aim being to search for functional mutations in the 5' flanking region beyond –3.4 kb (counting +1 as the start of translation), which was the point at the most 5' of *CYP1A2* to which Nakajima *et al.*, 1994 had screened). The GenomeWalker Kit provides a means of "walking" upstream or downstream in genomic DNA from a known sequence. The kit contains five "libraries" of uncloned genomic DNA fragments, produced by digestion of pure samples of high molecular weight genomic DNA by five different REs, *Eco*R V, *Sca* I, *Dra* I, *Pvu* II, and *Ssp* I, and which are then ligated to a 5'-extended 48-bp adaptor sequence (Figure 2.7). The process involves a 2-stage, or nested PCR, the primary PCR reaction utilising adaptor primer 1 (AP1) provided in the kit, and a gene-specific primer designed by the researcher. The 5' adaptor sequence has no binding site for the AP1 primer, and an AP1 binding site can therefore only be generated by polymerase-mediated extension from the gene-specific primer. In the secondary PCR reaction, the product of the first reaction is diluted and used as the template, with primers nested adaptor primer (AP2) and the nested gene-specific primer designed by the researcher (the nested gene-specific primer being further in the direction being travelled, *e.g.* further 5' in the known sequence). This generally produces a single, major PCR product from at least 4 out of the 5 libraries, and each of the 5 DNA fragments, which begin at the known sequence at the 5' end of the first gene-specific primer and extend into unknown adjacent genomic DNA, can then be analysed. The kit uses long PCR technology, and recommends the use of the r*Tth* supplied in the GeneAmp XL kit, but I compared the results with this and with the *Taq*/*Pwo* supplied in the Expand Long Template PCR kit, and found that the results with the *Taq*/*Pwo* were better. The gene-specific primers are

Genome Walker Adaptor



Adaptor primer 1 (AP1; 22-mer)

5'-GTAATACGACTCACTATAGGGC-3'

Nested Adaptor primer 2 (AP2; 19-mer)

5'-ACTATAGGGCACGCGTGGT-3'

Figure 2.7 Structure of the Genome Walker adaptor and adaptor primers. The adaptor is ligated to both ends of the genomic DNA fragments in all five Genome Walker "libraries" supplied with the kit. The amine group on the lower strand of the adaptor blocks extension of the 3' end of the adaptor-ligated genomic fragments, and thus prevents formation of an AP1 binding site on the general population of fragments. The design of the adaptor and adaptor primers is critical for the suppression PCR effect; the T_ms of AP1 and AP2 are 59^0C and 71^0C (from the GenomeWalker Kit user manual, Clontech).

recommended to be 25-28 nucleotides in length, with a G/C content of 40-60%, T_m greater than 68^0C, and 3' sequence that is not complementary to the 3' ends of the adaptor primers. In both cycling reactions, "touchdown" PCR is employed, involving using an annealing/extension temperature that is several degrees higher than the T_m of the primers during the initial PCR cycles, in order to increase specificity of annealing. The annealing/extension temperature is then reduced to slightly below the primer T_m for the remaining PCR cycles. Positive control gene-specific primers, specific for the human tissue-type plasminogen activator (tPA) locus, are supplied with the kit.

A primer specific for the known 5' flanking region of *CYP1A2*, towards the end of the published sequence (Quattrochi and Tukey, 1989), and GenBank sequence UO2993), was designed for use in the primary reaction: cyp1abr1, 5'-GGTTAGGTGCCATTCTCGTCACATC-3', using the recommended constraints regarding primer choice (as above). This primer was used in the primary PCR reaction as per the manufacturer's instructions, using the following cycling conditions in a MJ Research tetrad PCR machine (as the manufacturer recommended for a Perkin-Elmer GeneAmp PCR 9600 cycler, with the addition of an initial denaturation step for 2 min): initial denaturation at 94^0C for 2 min; followed by 7 cycles of 94^0C for 2 s, 72^0C for 3 min; followed by 32 cycles of 94^0C for 3 s, 67^0C for 3min; and ending with a terminal elongation step of 67^0C for 4 min. A pseudohotstart was used, placing the samples in the block when it had reached 80^0C. The reaction products were run on a 1.5% agarose gel, and a clean band was seen in the lane from library number 3 (*Dra* I library). A second, nested, gene-specific primer was designed further 5', cyp1abr2,. 5'-GTGCGTGTCAGGTCTCTTCACTGTA-3', and used together with AP2 with the products from each of the libraries in the first PCR reaction, with the following cycling

conditions (again, MJ Research machine, minor modifications to the protocol given by the manufacturer for use in a 9600 cycler): initial denaturation at 94^{0}C for 1.5 min; followed by 5 cycles of 94^{0}C for 2 s, 72^{0}C for 3 min; followed by 20 cycles of 94^{0}C for 3 s, 67^{0}C for 3 min; and ending with a terminal elongation step of 67^{0}C for 4 min. Again, a pseudohotstart was performed by placing the samples in the block once it had reached 80^{0}C. This time bands were seen in the lanes from all libraries on the agarose gel, with the bands being particularly clean in the lanes of libraries 2 and 4 (*Sca* I and *Pvu* II libraries, Figure 2.8). These second PCR products were purified using QIAquick PCR purification (see below), and sequenced directly using 60-90 ng of purified PCR product, standard cycle sequencing with primer cyp1abr2, ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (see below), and an ABI 377 DNA sequencer.

2.2.7 Techniques relating to automated sequencing

2.2.7.1 PCR product purification

PCR products were purified using the QIAquick PCR Purification Kit (Qiagen Ltd., UK), to eliminate the primers, dNTPs, salts and polymerase from the PCR reaction. Five volumes of buffer PB (containing chaotropic salts; the composition of the individual buffers is not given by the manufacturer) were added to 1 volume of PCR product and mixed by vortexing. The mixture was applied to a QIAquick spin column within a 2 ml collection tube and centrifuged at 13,000 rpm (>10,000 x *g*) in a microcentrifuge for 1 min (all centrifuge steps were conducted at this speed). During this step, the DNA adsorbs to the silica-gel membrane within the column (DNA adsorption requires a pH <7.5 and high salt concentration, which is provided by the

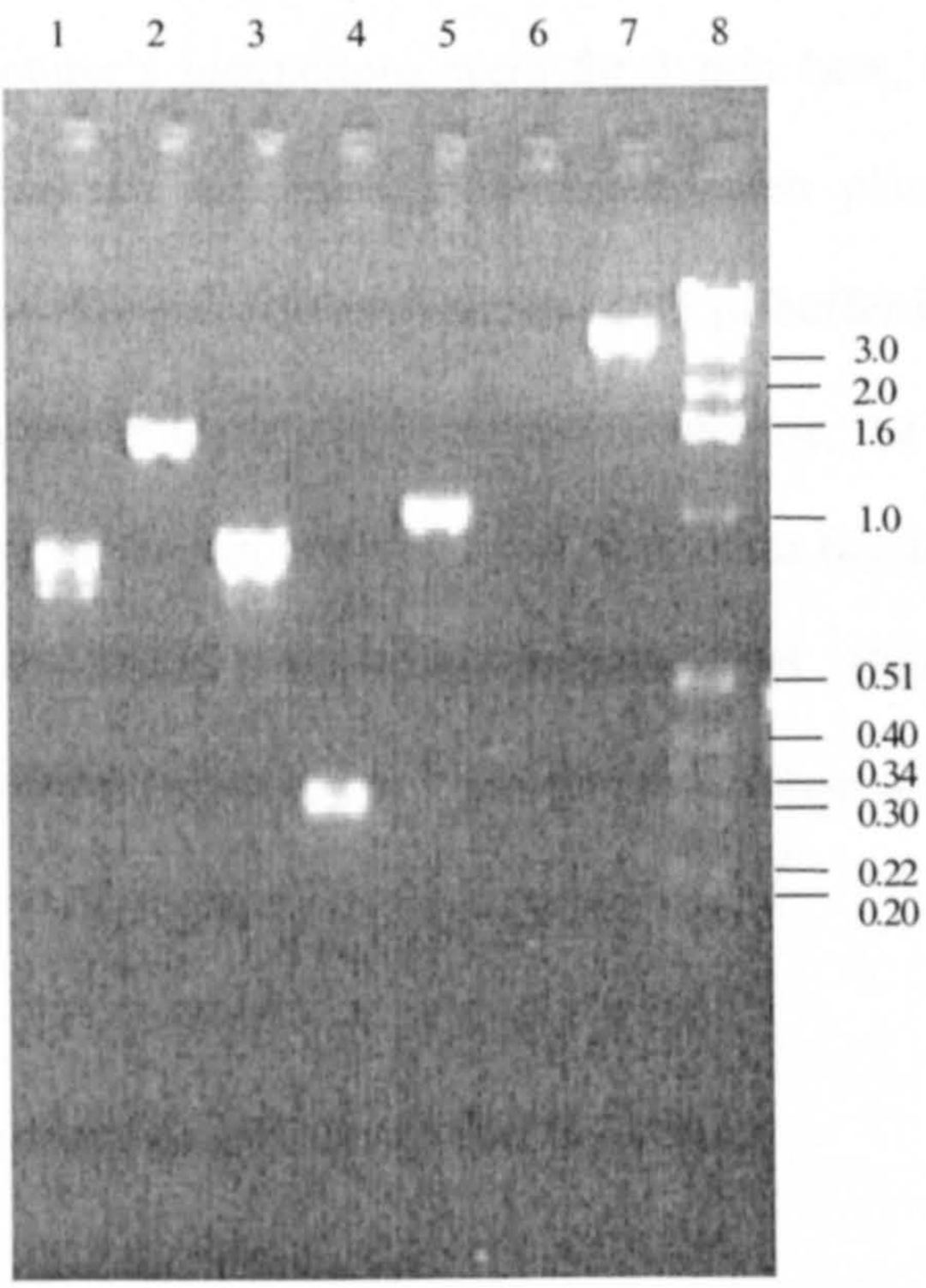


Figure 2.8 Agarose gel electrophoresis of products of secondary PCR using the GenomeWalker kit and primers cyp1abr2 and AP2. Lane 1: *Eco* RV library; lane 2 *Sca* I library; lane 3: *Dra* I library; lane 4: *Pvu* II library; lane 5:*Ssp* I library; lane 6: negative control; lane 7: positive control supplied with kit; lane 8: 1kb ladder (Gibco BRL, Inc., kb).

addition of buffer PB), whilst the excess PCR reagents flow through to the collection tube and are discarded. The DNA bound to the silica-gel was then washed by the application of 0.75 ml of buffer PE, followed by centrifugation for 1 min. The flow-through was discarded, and the spin column centrifuged for 3 min to remove residual buffer (the manufacturer's instructions were for 1 min here, but I found that 3 min produced better results). The spin column was then placed in a clean 1.5 ml microcentrifuge tube. In order to elute the DNA, 25 µl buffer EB (10 mM Tris.Cl, pH 8.5) was applied to the centre of the QIAquick membrane, the column was allowed to stand for 3 min, and then spun at 13,000 rpm for 3 min (again, this represents minor modifications of the protocol given by the manufacturer). (For the work conducted at NIH, USA, the Wizard PCR Preps DNA purification system was used, which works on similar principles.)

2.2.7.2 Automated sequencing

2.2.7.2.1 Cycle sequencing of PCR products

Cycle sequencing using fluorescently-labelled terminators is a rapid and convenient method for performing enzymatic extension reactions for DNA sequencing. The ABI Prism™ dRhodamine cycle sequencing ready reaction kit (Applied Biosystems, Perkin Elmer) contains the sequencing enzyme *AmpliTaq* DNA polymerase FS, dye terminators, dNTPs, $MgCl_2$, and buffer pre-mixed in a single tube which is stored at – 20^0C until use. The concentration of purified PCR product was first estimated by running 1 µl on a 1% agarose gel (1 µl purified product, 1 µl 6 x loading buffer, and 3 µl Tris, the latter to enhance the result using a low sample volume), alongside standards of known concentration (*e.g.* a plasmid, pTET, of known concentration, supplied by A.

J. Makoff, or DNA Quantitation Standards, GibcoBRL), judging concentration of the PCR product by comparision, by eye. For PCR products of less than 1 kb, 30-90 ng is suitable for cycle sequencing, and so the above rough estimation is sufficiently accurate. (The amount of PCR product required for good results on cycle sequencing depends on the length; products of 1-2 kb, and greater than 2 kb require 100-200 ng and 200-300 ng respectively.) The following sequencing reaction was then set up for each sample: 6 μl terminator ready reaction mix, x μl PCR product equivalent to 30-90 ng, 1 μl of sequencing primer at 3.2 pM, and PCR-grade H_2O to make the total volume up to 20 μl. Alternatively, in order to conserve the expensive terminator ready reaction mix, reactions with half total volume were set up, using half the above quantities, except for the terminator ready reaction mix at 4 μl. If the Perkin Elmer DNA Thermal Cycler was used, the reactions were set up in 0.5 ml microcentrifuge tubes, and overlaid with 40 μl mineral oil, with cycling conditions of 25 cycles of 96^0C for 30s, 50^0C for 15 s, and 60^0C for 4 min, preceded by a 95^0C for 3 min during which the samples were added to the machine once it had reached 95^0C, and followed by a 4^0C hold. If the Perkin Elmer 9600 cycler was used, reactions were set up in 96-well plates, and the heated lid option was employed, with the following cycling conditions: 25 cycles at 96^0C for 10 s, 50^0C for 5 s, 60^0C for 4 min, followed by a 4^0C hold (following the manufacturer's instructions).

2.2.7.2.2. Cycle sequencing of BAC clones

Direct sequencing of BAC clones was similarly performed using cycle sequencing. If the dRhodamine cycle sequencing ready reaction kit was employed, then the following protocol was used: 22 μl of BAC DNA at 2 μg/μl, 16 μl of terminator ready reaction mix, with 2 μl of primer at 3.2 pmol/μl, and cycle sequencing conditions of: 50 cycles

of 95^{0}C for 30 s, 50-55^{0}C (depending on primer T_m) for 20 s, and 60^{0}C for 4 minutes. Or, alternatively, the ABI Prism^{TM} BigDye Terminator Kit was used, with the following protocol: 8 µl BAC DNA to give a final amount of 1-1.5 µg, 8 µl Big Dye terminator mix, 1 µl of primer at 25 pmol/µl, and 3 µl of PCR-grade H_20. The cycling conditions employed for the latter were the same as with the dRhodamine kit, with 55^{0}C for 20 s as the second step producing good results with the primers used. It was found that a hot start was not required for BAC sequencing, and results were best when only 1.5-2.0 µl of sequencing buffer was used per sample.

2.2.7.2.3 Preparation of samples for the sequencer

2.2.7.2.3.1 Column purification

In order to eliminate unincorporated fluorescent dideoxynucleotides (that would produce background fluorescence on the sequencing gel), the extension products of the cycle sequencing reaction were purified prior to loading on the sequencing gel. For the work conducted at NIH, USA, a column purification method was used, employing either Centri-sep columns or columns made by Edge Biosystems, Inc (Gaithersburg, MD, USA). The protocol for the latter was as follows: the supplied unit (cartridge and microtube) were placed in a microcentrifuge and centrifuged at 750 x *g* for 1 min (= 3.0 x 1000 rpm on an Eppendorf microcentrifuge). If the Thermal Cycler had been used, the products of the cycle sequencing reaction were then de-oiled (by decanting the samples on to parafilm, and lifting up the parafilm carefully so that the samples ran down the parafilm, leaving the oil behind, and recovering the de-oiled sample). The cartridge was then transferred to a clean microtube (provided with the kit), and the sample added to the centre of the gel surface. The cap of the microtube was then

closed and the tube with contents centrifuged at 750 x *g* for 2 min. The eluate was then retained, and dried (by placing in a speedivac for 45 min). Samples were then prepared for loading on the sequencing gel by the addition of 4 µl sequencing buffer, followed by centrifugation, placing in an eppendorf shaker for 15 minutes, and denaturing by heating to 95^{0}C for 5 min with immediate cooling on ice.

2.2.7.2.3.2 Ethanol precipitation

For the work that was conducted in the UK, the extension products were purified by ethanol precipitation. To a 1.5 ml microcentrifuge tube, 2 µl of 3M sodium acetate at pH 4.6 and 50 µl 95% ethanol was added. The sample (de-oiled as above, if necessary) was added to this, the mixture vortexed, and then left at RT for 20 min to precipitate the DNA. The microcentrifuge was then centrifuged at 13,000 rpm for 20 min (with the hinge of the microcentrifuge to the periphery, so that the position of the DNA precipitate can be located to the bottom of the microcentrifuge tube, beneath the hinge), and the supernatant carefully aspirated. The pellet was then respun, and the rest of the supernatant carefully removed. The resulting pellet was then rinsed with 250 µl of 75% ethanol, vortexed briefly, and recentrifuged at 13,000 rpm for 10 min. At this point all the ethanol was then carefully removed. The pellet was then dried, either by leaving to dry in air at RT for at least 30 min, or by drying in a microcentrifuge with the microcentrifuge lid cut off. Samples were then prepared for loading on the sequencing gel as above.

2.2.7.2.4 Use of the ABI Prism 377 DNA fluorescent sequencer

Electrophoresis on the ABI Prism 377 DNA fluorescent sequencer was conducted according to the manufacturer's protocol (Applied Biosystems, Perkin Elmer). In

summary, a 38-well denaturing polyacrylamide gel (4.25%, with 35 µl TEMED) was poured in between the sequencing plates, taking care to exclude air bubbles, left to set, excess gel was removed, and the comb was inserted to a depth of approximately 3 mm. The plates with gel were then placed in the sequencer, scanned for background fluorescence, the tanks in the sequencer were filled with 1x TBE buffer, and a pre-run at 48^{0}C for 30 min was conducted. The prepared samples (1.8 or 2 µl) were then loaded into each well, loading every other well initially, and electrophoresing the gel for 2 min prior to loading the remaining samples. Electrophoresis was then conducted for 7 hours. Raw data for each sample was generated and analysed using the ABI 377XL collection (v2.4) and Sequencing Analysis (v.3.2) software. The resulting electropherograms were analysed by hand and by use of software packages including MacVector (Oxford Molecular Limited, Oxford, UK), Hitachi MacDNASIS Pro version 3.4 (Novex, San Diego, CA), and GeneTool lite version 1.0 (DoubleTwist, Inc., CA, USA).

2.2.8 Techniques using DNA cloning

2.2.8.1 Cloning and sequencing of PCR products

PCR products chosen for cloning were cloned using the TA Cloning Kit (Invitrogen Corporation, Carlsbad, CA, USA). The principle of the TA Cloning Kit is that *Taq* polymerase has a non-template-dependent activity which adds a single deoxyadenosine (A) to the 3' ends of the PCR product; the linearized vector (pCR2.1) supplied with the Kit has single 3' deoxythymidine (T) residues, therefore allowing efficient ligation of the PCR inserts with the vector. The vector also contains a *lac* promoter and *lacZα*

fragment (encoding the first 146 amino acids of β-galactosidase) for the "blue-white screening" method of identification of colonies containing the PCR insert (white colonies containing the insert). The site of insertion into the vector is in between the *lac* promoter and the *lacZ*α fragment. The vector also contains a T7 priming site and M13 forward and reverse priming sites, to facilitate sequencing of an insert.

For the use of this technique, I found that it was important to end the PCR cycling reaction with a final 7-10 minute extension step, but even with this, if the sample was more than 24 hours old when cloned, the 3'-A overhangs were readded as follows: to 25 µl of PCR product was added 5 µl of 10 x PCR reaction buffer from the kit (contains $MgCl_2$ to a final concentration of 2.5mM), 1 µl 10mM dATP (to a final concentration of 0.2mM), 0.5 µl Taq (5U/µl Life Technologies, Inc, Gaithersburg, MD, USA), and 18.5 µl of PCR-grade water, and incubation at 72^{0}C for 10 min followed by cooling to RT was conducted. The procedure for TA cloning into the vector pCR2.1 was then followed as per the manufacturer's instructions (except that 3 ligation reactions were set up, see below). The following formula was used to estimate the amount of PCR product needed to ligate with 50 ng (20 fmoles) of pCR2.1 vector:

$$\text{X ng PCR product} = \frac{\text{(Y bp PCR product)(50 ng pCR2.1 vector)}}{\text{(size in bp of the pCR2.1 vector: approx 3900 bp)}}$$

Three ligation reactions were then set up as follows, one with a ratio of vector to insert of 1:1 (ng:ng), one with a ratio of 1:3, and one with no insert (as a control): x µl PCR product (corresponding to the correct ng PCR product, calculated as above), 1 µl 10 x ligation buffer (supplied in the kit), 2 µl pCR2.1 at 25 ng/µl, 1 µl T4 DNA ligase (equivalent to 4 Weiss units, or 240 cohesive end units), and sterile water to a total

volume of 10 µl. The ligation reaction was then incubated at 14^{0}C in a water bath overnight.

Instead of the competent cells recommended in the TA Cloning Kit, the lab's own competent cells were used (I conducted these experiments in Denver, Colorado; the cells were made by Linda Quattrochi's staff; *E. coli* strain MN 522, which work best with pBluescript derived plasmids). These cells express the *lac* repressor, which will repress transcription from the *lac* promoter, unless IPTG is added. Plates of Circle Grow (CG) and LB agar containing 50 µg/ml of ampicillin were warmed at 37^{0}C for 10 min, then 80 µl of 20 mg/ml X-Gal and 40 µl 100 mM IPTG was spread evenly onto the plates using a sterile spreader, and allowed to diffuse into the plates by incubating at 37^{0}C for a further 20-30 min. The reaction tubes containing the ligations were then centrifuged briefly and placed on ice. The competent cells were thawed on ice, and 2 µl of each ligation reaction was pipetted directly into the competent cells, and mixed by stirring gently with a pipette tip. The vials were then incubated on ice for 30 min (and the remainder of the ligation reactions stored at –20^{0}C), following which they were heat shocked for exactly 30 s in a 42^{0}C water bath, then placed on ice for 2 min. At this point 250 µl SOC medium at RT (Table 2.1) was added, and the vials were shaken horizontally at 37^{0}C for 1 hour at 225 rpm in a rotary shaking incubator. The vials with the transformed cells were then placed on ice. They were plated out on to the prepared plates as follows: 50 µl and 200 µl from each transformation reaction was plated on to each prepared CG and LB plate respectively (as CG agar tends to give a higher density of growth). A control transformation using pUC18 (1 µl of pUC18 diluted to 10 pg/µl) was also conducted. Once the liquid was absorbed, plates were incubated at 37^{0}C for 18 hours, then shifted to 4^{0}C for 2-3 hours to allow for proper colour development.

Blue and white colonies were obtained, and also some white colonies with blue centres as the insert was shorter than 500 bp and therefore achieved only partial disruption of *lac*Z in some cases. The protocol for colony lysis and PCR was then followed from the PCR-Trap manual (GenHunter Corporation, Nashville, TN, USA). On the back of each plate, each colony to be analysed (white or white with blue centre) was numbered, and then picked with a sterile toothpick and placed in 50 µl colony lysis buffer in a microcentrifuge tube. The tubes were then incubated at 95^0C for 10 min, centrifuged for 2 min to pellet the cell debris, and the supernatant was transferred to a clean tube and used immediately for PCR analysis or stored at -20^0C. Primers M13R (5'-CAGGAAACAGCTATGAC-3') and T7 (5'-TAATACGACTCACTATAGGG-3') were used at a final concentration of 0.2 µM in a total reaction volume of 20 µl with 2 µl of colony lysate, 20 µM dNTPs, 1x PCR buffer including $MgCl_2$ (concentration not given by manufacturer), and 1 U *Taq*. Cycling conditions were as follows: initial denaturation at 94^0C for 30 s; 30 cycles of 94^0C for 30 s, 50^0C for 30 s, 72^0C for 45 s; and a terminal elongation step of 72^0C for 7 min. PCR products were then analysed on a 3% agarose gel, with those positive for an insert being clearly identifiable (being 264 bp or 273 bp longer than the negative clones for the products of the cyp1abr2-M5 and M3-M4 PCRs respectively). One of the samples cloned in this way (Black American sample number 9) generated four positive clones thus identified, which were subjected to standard cycle sequencing using T7 as a sequencing primer. The second sample cloned in this way (Black American sample number 43) generated 35 colonies positive for an insert. As this sample had already been identified to be heterozygous for the T.$_{3591}$G substitution, which generated an *Mbo* II restriction site, we were able to screen these PCR products using *Mbo* II digestion (using 10 µl PCR product, 1.5 U *Mbo* II,

and making up the volume to a total volume of 50 µl with 34.7 µl PCR-grade water). Products of colony PCR which digested with *Mbo* II and products which did not were then subjected to sequencing, again using T7 as a sequencing primer.

2.2.8.2 Site-directed mutagenesis

Site-directed mutagenesis was performed using the QuikChange™ Site-directed mutagenesis kit (Stratagene Inc., La Jolla, CA, USA) on the pL1A2N plasmid that had already been constructed by LQ. This plasmid is approximately 10kb in length (double-stranded), and contains a *Kpn* I fragment of *CYP1A2* (-3201/+53) proximal to a luciferase reporter gene (Postlind *et al.*, 1993; Figure 2.9). (This plasmid also contains an SV40 promotor and a neomycin resistant gene, which were not necessary for the purpose of transient transfections, but this plasmid was used as it was already in LQ's possession and had been previously used to generate stable transfectants.) The QuikChange™ site-directed mutagenesis method can be used to make point mutations, switch amino acids, and delete or insert single or multiple amino acids in a double-stranded plasmid, generating mutants with an efficiency of at least 80%, using *PfuTurbo*™ DNA polymerase (which has a 6-fold higher fidelity than *Taq* DNA polymerase). Two primers containing the desired mutation are extended during temperature cycling by *PfuTurbo* DNA polymerase, generating a mutated plasmid with staggered nicks (Figure 2.10). The product of cycling was then treated with *Dpn* I (target sequence 5'-Gm6ATC-3'), which is specific for methylated and hemimethylated DNA, and therefore digests the parental (non-mutant) DNA, selecting for mutation-containing synthesised DNA. The nicked vector DNA incorporating the desired mutations is then transformed in to Epicurian Coli XL1-Blue supercompetent cells. The small amount of the starting DNA template required to perform this method, the

high fidelity of the *PfuTurbo* DNA polymerase, and the low number of PCR cycles all contribute to the high mutation efficiency and decreased potential for random mutations during the reaction.

Site-directed mutagenesis to create the $T_{-3591}G$ mutation was performed with primers SDM1F (5'-CCTGTAATTTAATTTTTTTAAGTTTGAAGAAAACATTAAAAATAAAAAG-3') and SDM1R (5'-CTTTTTATTTTTAATGTTTTCTTCAAACTTAAAAAAATTAAATTACAGG-3'), where the underlined nucleotides are the sites in the primers that differ from the wild-type sequence, and constraints regarding primer design were followed as in the manufacturer's instructions. Reaction conditions were as in the manual (with 125 ng of each primer and 1 μl of *Pfu* (2.5 U/μl) set up in thin-walled PCR tubes and overlaid with 30 μl of mineral oil), 4 different reactions being set up, using 5 ng, 10 ng, 20 ng, and 30 ng of plasmid. Cycling conditions were: initial denaturation of 95^{0}C for 30 s;18 cycles of 95^{0}C for 30 s, 55^{0}C for 1 min, and 68^{0}C for 20 min; followed by cooling to 4^{0}C. Cycling conditions for the control reaction were as in the manual (*i.e.* initial denaturation of 95^{0}C for 30 s; 12 cycles of 95^{0}C for 30 s, 55^{0}C for 1 min, 68^{0}C for 12 min; followed by cooling to 4^{0}C). When the products were run on a 1% agarose gel, a clean band was visible in the lanes of the 20 ng and 30 ng reactions. *Dpn*I digestion of these products was then carried out as per the manual, and Epicurian Blue cells transformed as per the manufacturer's instructions. For the creation of Xgal/IPTG plates (for the control and pUC18 reactions), 50μl of 20mg/ml Xgal (in DMF) was spread on to the plates, left to soak in, and then 75 μl of 25mg/ml IPTG was spread on

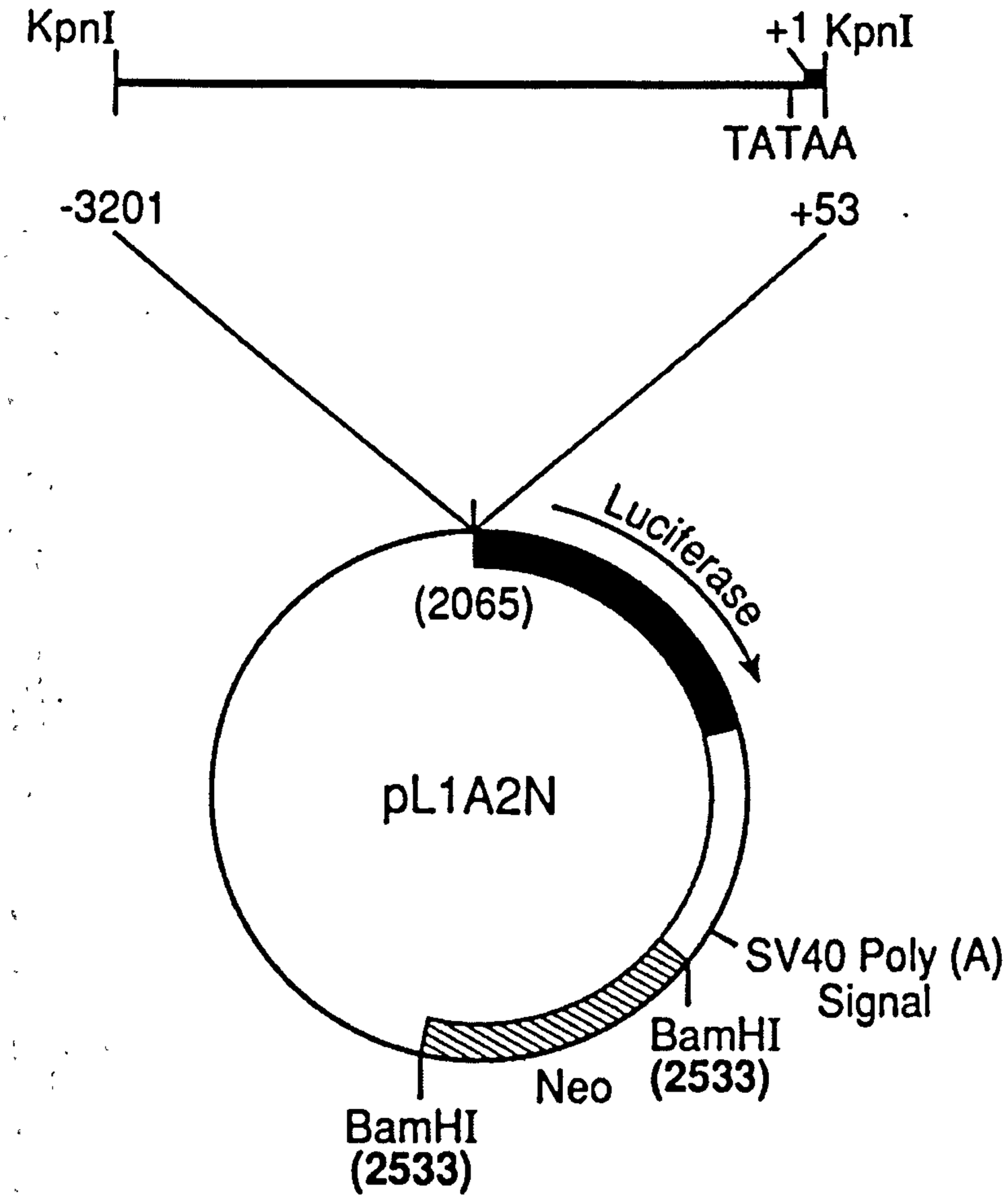


Figure 2.9 Schematic map of the pL1A2N expression vector, containing a *Kpn* I fragment of *CYP1A2* (-3201/+53), that includes exon 1 (black box), no intronic sequence, and 3201 bases of human *CYP1A2* 5' flanking sequence, proximal to a luciferase reporter gene. The start of transcription is indicated by +1 and the consensus TATAA sequence is indicated. The coordinate, 2065, corresponds to the unique *Hind* III site of the pSVO fector (De Wet *et al.*, 1987) and coordinates in bold correspond to the location of the restriction sites in the published sequence of SV40 (Buchman *et al.*, 1981). From Postlind *et al.* (1993).

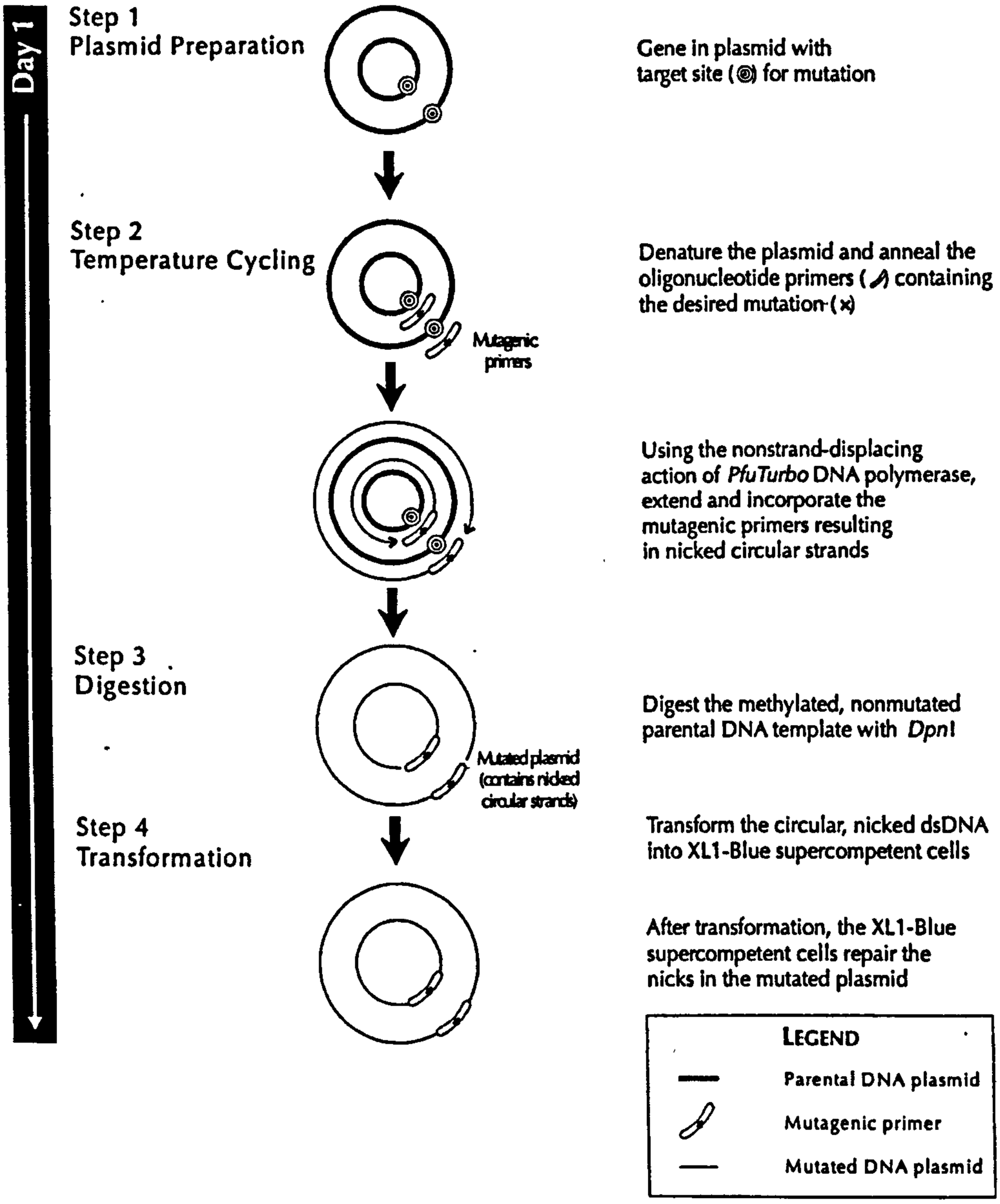


Figure 2.10 Overview of the QuikChange™ site-directed mutagenesis method (from the QuikChange™ Site-Directed Mutagenesis Kit Instruction Manual).

the plates. All 500 µl of the sample reactions were plated on to CircleGrow (BIO 101, Inc, Vista, CA) agar plates with ampicillin at 50 ng/ml, and the pUC18 control reaction was plated in NZY+ broth as per the manual. Plates were then incubated at 37^0C for 16 hours. Approximately 1000 colonies resulted on the sample plates, with the control plates showing a mutagenesis efficiency of approximately 95%, and the pUC18 plates revealing a high transformation efficiency (>10^8 cfu) with >98% having the blue (β-gal+) phenotype. Single colonies were then picked from the sample plates, and plasmid DNA isolated as described above (using ampicillin). DNA was then quantified using a spectrophotometer (absorbance at 260 nm).

Following creation of the mutant plasmid by the above procedure, the plasmid was subjected to sequencing using primer M3. Sequencing confirmed correct introduction of the mutation, and absence of any other differences in DNA sequence from the parent plasmid. Sequence comparison was undertaken with the programme Hitachi MacDNASIS Pro version 3.4 (Novex, San Diego, CA). This plasmid was then termed SDM1.

Site-directed mutagenesis to create the $G_{-3595}T$ mutation was performed in a similar manner to the above, using primers SDM2F (5'-CTATATTGTATCCTGTAATTTAATTTTTTTAAT<u>T</u>TTTTAAGAAAAC-3') and SDM2R (5'-GTTTTCTTAAAA<u>A</u>TTAAAAAAATTAAATTACAGGATACAATATAG-3'). This time, reactions were set up using 10 ng, 20 ng, 30 ng, and 40 ng of plasmid, and clear bands resulted from each, using the same cycling conditions as for the creation of SDM1. The 10 ng reaction was used for *Dpn*I digestion, and 1.5 µl of *Dpn*I was used

(as the PCR product was of greater intensity than for SDM1, and I wanted to ensure complete digestion of parent plasmid). Transformation and plasmid preparation was then undertaken as for SDM1, and, again, sequencing was performed with primer M3 to ensure correct introduction of the mutation. The resultant plasmid was termed SDM2.

A third site-directed mutagenesis reaction was performed in order to create a double mutant, with both the $T_{-3591}G$ and the $G_{-3595}T$ substitutions, using primers SMD3F (5'-CTATATTGTATCCTGTAATTTAATTTTTTTAATTTTGAAGAAAAC-3') and SDM3R (5'-GTTTTCTTCAAAATTAAAAAAATTAAATTACAGGATACAATATAG-3'). This time the transformation reactions were split into 2 before plating, and fewer, larger colonies resulted. Plasmid preparation and confirmation of mutagenesis by sequencing were then conducted as for SDM1, and the resultant, correctly mutated plasmid was termed SDM3.

2.2.9 Transient transfections

HepG2 cells were seeded at 1.25×10^5 /ml in 6-well plates in complete growth medium with 10% serum. The complete growth medium consisted of Dulbecco's Modified Eagle Medium and F-12 Nutrient Mix in a 1:1 ratio (DMEM/F12, 1:1, GIBCO BRL, Life Technologies), with 10 mM Hepes, 100 U/ml penicillin, and 100 ug/ml streptomycin. Cells were grown up for 24 hours at 37^0C under 5% CO_2, and transfected the next day with 2μg plasmid and 0.5 μg CMVβ (ClonTech Laboratories Inc., Palo Alto, CA), using lipofectamine reagent (Life Technologies, Inc., Gaithersburg, MD, USA), with a 1:8 ratio (mass:mass) of DNA:lipofectamine and OPTI-MEM I (Life

Technologies, Inc., Gaithersburg, MD, USA) as the serum-free medium. The CMVβ plasmid expresses β-galactosidase from the human cytomegalovirus immediate early gene promotor. For each transfection, a minimum of eight 6-well plates were set up, 2 being transfected with pL1A2N, 2 with SDM1, 2 with SDM2, and 2 with SDM3 (and each being cotransfected with the β-galactosidase plasmid). The DNA, lipofectamine, and OPTI-MEM solution was incubated at room temperature for 1 hour to allow complexes to form, the cells were overlaid with 1ml of complex-containing solution per well, and the plates were incubated at 37^{0}C under 5% CO_2 for 5 hours. The complex-containing solution was then removed, and replaced with 1ml prewarmed 10% A2. The next day the medium was replaced with fresh complete medium, and at 40 hours post transfection 1 plate for each plasmid was treated with 10nM TCDD (a CYP1A2 inducer), and for the other plate the medium was replaced with fresh complete medium. Sixty hours post transfection, the cells were harvested for luciferase activity, using a Luciferase Assay System with Reporter Lysis Buffer (Promega Corporation, Madison, WI, USA), 200μl Reporter Lysis Buffer per well. The lysates were cleared by centrifugation for 2 min at 4^{0}C, and 20 μl of cleared lysate was then assayed for luciferase activity using a Lumat LB9501 luminometer (Berthoid, Sci West, Arvada, CO, USA). The remaining cleared lysates were then frozen at –70^{0}C. The next day the lysates were assayed for β-galactosidase activity using the β-galactosidase Enzyme Assay System with Reporter Lysis Buffer (Promega Corporation, Madison, WI, USA), using a microtitre plate (Nalge NUNC International, Naperville, IL, USA) for the 96-well format and preparing a standard curve using the β-galactosidase supplied. Lysates (50 μl, undiluted) and standards were assayed in duplicate. After the addition of Assay 2x Buffer, plates were incubated at room temperature for 15 min before the first absorbance reading in a plate reader at 410 nm, with 490 nm as a comparative

wavelength. Serial readings were then taken every 15 min until an hour after the first reading (so that the best linear standard curve could be elicited)..

2.2.10 Studies with wild-type and CYP1A2-null mice

2.2.10.1 Study design

Human and mouse CYP1A2 resemble each other closely in cDNA derived amino acid sequence (Kimura *et al.*, 1984; Jaiswal *et al.*, 1987) and in catalytic activity (Aoyama *et al.*, 1989). Given the inconsistencies in the conclusions of previous studies regarding the contribution of CYP1A2 to clozapine metabolism (Section 5.1), my aim was to use the *CYP1A2* -/- (null) mouse in order to investigate the *in vivo* contribution of CYP1A2 to clozapine pharmacokinetics. In addition, I used the *CYP1A2* -/- mouse as a model for individuals with relatively low CYP1A2 activity, through the use of behavioural ratings aiming to draw conclusions regarding the pharmacodynamic effects of clozapine in such individuals.

Clozapine was purchased from Sigma Chemicals Co. (St Louis, MO, USA), and desmethylclozapine and clozapine *N*-oxide from Research Biochemicals International (Natick, MA, USA). All other chemicals were of analytical-grade, available from commercial sources. A clozapine solution of 1mg/ml was made (clozapine powder was dissolved in 0.1M HCl, neutralised to pH 5-6 with 1M NaOH, and made up to 1mg/ml with deionised distilled water). All mice were weighed, and at time = 0, a dose of 10 mg/kg clozapine was given intraperitoneally. I was taught how to administer intraperitoneal injections to the mice by Dr Takafumi Sakai (a visiting fellow at Dr

Gonazelez's laboratory during the same time period that I was there). Eight sequential blood samples (each approximately 50 µl) per mouse were then taken by tail blood sampling at 5, 15, 30, 60, 120, 240, 360, and 480 min post injection (the injection times of the mice were approximately 20 min apart for ease of sampling). The method of tail blood sampling was by sequential clipping of the tail (2-3 mm of tail being cut at each interval), followed by cauterisation to stop bleeding. The blood was collected in heparinised tubes (Sarstedt Ltd, Leicester, UK), and stored at -80^{0}C until analysis. Behavioural parameters (degree of drowsiness, motor incoordination) were recorded at each time point. After the last sample had been taken, the mice were killed by carbon dioxide asphyxiation.

2.2.10.2 Behavioural effects ratings

In the pilot studies, I noticed that clozapine at the dose administered caused drowsiness and motor impairment in the mice. I therefore devised a scale for the rating of these effects (Table 2.3), based on repeated observations of characteristic motor and behavioural changes of wild-type and CYP1A2 -/- mice during pilot studies with clozapine. Mice were scored at each time point, just before tail blood sampling.

2.2.10.3 HPLC analysis

Clozapine, desmethylclozapine, and clozapine *N*-oxide in the blood samples were analysed by HPLC with UV detection at 254 nm as described (Volpicelli *et al.*, 1993), by Prof Michael W. Jann (Mercer University Pharmacy Practice, Atlanta, GA, USA). The recovery from control rat serum at 500 ng/ml of clozapine, 500 ng/ml of desmethylclozapine, and 100 ng/ml of clozapine *N*-oxide was 78%, 84%, and 62% respectively (N = 6 samples). A standard assay curve was completed and repeated six

times using 7 data points for clozapine of known concentrations in the range 10 ng/ml to 1000 ng/ml ($r^2 = 0.996$). The lower limit of detection was 5.0 ng/ml for clozapine, desmethylclozapine, and clozapine *N*-oxide, and the interassay and intraassay coefficients of variation (CV) were 8.2% and 5.0% respectively for all three substances.

2.3 Data analysis

Data analysis was conducted using SPSS version 8.0 for Windows (Statistical Package for the Social Sciences, Chicago, IL, USA), EpiInfo version 6.0 (Centers for Disease Control and Prevention, USA and World Health Organization, Switzerland), Solo **Table**

Table 2.3 Scale for rating behavioural effects of clozapine in mice

Clozapine behavioural effects ratings in mice
Drowsiness
0 Fully alert
1 Eyes half closed at rest
2 Eyes closed at rest, but easily rousable
3 Eyes closed at rest, difficult to rouse
Motor impairment
0 Normal posture at rest, move normally in cage on handling
1 Mild splaying of legs at rest, movements slowed and jerky, mild reduction in struggle on handling
2 Moderate splaying of legs at rest, little movement in cage, moderate reduction in struggle on handling
3 Prominent splaying of legs at rest (sprawled), no movement in cage, minimal struggle on handling

Power Analysis (BMDP Statistical Software, Los Angeles, CA, USA), NCSS 2000 (Statistical analysis and data analysis software, Kaysville, UT, USA), and Axum version 5.0 (MathSoft, Inc., Cambridge, MA, USA). A *P* value of less than 0.05 was interpreted as significant unless a Bonferroni correction was being applied.

2.3.1 Calculation of metabolic ratios and phenotype assignment

The debrisoquine MR (MR_{DBQ}) was defined as the molar ratio of the concentrations of debrisoquine to 4-hydroxydebrisoquine in an 8-hour overnight urine collection (Maghoub *et al.*, 1977). The cut-off points between different phenotypes are defined by the antimodes on a log-normal distribution of the MRs (*i.e.* a plot of the frequency versus $logarithm_{10}$ of the MR). In Caucasians, the cut-point between CYP2D6 extensive metabolisers (EMs) and poor metabolisers (PMs) is usually defined as 12.6 (Evans *et al.*, 1980; Steiner *et al.*, 1985), although other antimodes have been described (7.28 by Evans *et al.*, 1983, on reanalysis of their original data, or 5.42 by the same authors in a separate group of 215 British white subjects, and 4.48 in a study of 127 unrelated Spaniards by Henthorn *et al.*, 1989). However, the numbers of individuals with MRs within the range 4.48-12.6 is predicted to be small, so a very small number of individuals would be alternatively classified with a different choice of cut-point (Henthorn *et al.*, 1989). Moreover, in the most comprehensive genotype-phenotype association study conducted in Caucasians (Sachse *et al.*, 1997), the antimode of 12.6 correctly classfied all PMs.

The ultrarapid metaboliser (UM) phenotype has been defined as an MR_{DBQ} less than 0.20 (Dahl *et al.*, 1995a). However, as has been noted by Løvlie and colleagues (2001), this cut-off point is arbitrarily defined, and some studies (e.g. Johansson *et al.*, 1996)

define UMs as those with $MR_{DBQ} < 0.15$. I have similarly analysed my data using both alternative cut-points, determining the genotype-phenotype correlations. The EM phenotype (including intermediate metabolisers, or IMs) corresponds to MR_{DBQ} lying between the cut-points for UMs and PMs.

Similarly, the dextromethorphan MR is defined as the molar ratio of the urinary concentrations of dextromethorphan to dextrorphan, with $MR_{dextromethorphan}>0.3$ defining CYP2D6 PMs (Schmid *et al.*, 1985; Larrey *et al.*, 1987). A genotype-phenotype correlation study in 456 German Caucasians phenotyped with dextromethorphan found that the mean $MR_{dextromethorphan}$ values (with 95% confidence limits of the mean) for individuals genotyped as having none, one, two, or three functional *CYP2D6* genes were 1.902 (1.392-2.598), 0.009 (0.007-0.012), 0.003 (0.002-0.003), and 0.002 (0.001-0.005) respectively (Sachse *et al.*, 1997).

2.3.2 Association analysis

2.3.2.1 Genotypic distribution

In the case of CYP2D6 analysis, genotyping was conducted to identify 3 non-functional alleles (*CYP2D6*3*, *CYP2D6*4*, and *CYP2D6*5*), and also the gene duplication event, and further assays (see section 2.2.3.2) were also performed, in order to identify duplications of non-functional alleles (*e.g. CYP2D6*4* duplications). It was therefore possible to identify the number of functional *CYP2D6* genes, or *CYP2D6* gene dosage, where this figure varied between 0 and 3, with a value of zero corresponding to homozygosity for a non-functional allele (including duplications thereof), a value of 1 indicating possession of one copy of an active (wild-type) allele, a value of 2 indicating

2 copies of an active (wild-type) allele, and 3 indicating at least 3 copies of an active *CYP2D6* allele. Where cases were positive on the duplication assay but negative for non-functional alleles, they were assumed to be heterozygous for the duplication allele, not homozygous, as in the paper by Johansson *et al.* (1996) zero out of 17 UM cases (debrisoquine MR <0.15) were homozygous for a duplication event. PMs correspond to those with a CYP2D6 gene dosage of 0, and UMs to a gene dosage of at least 3 (up to 13 according to the work of Johansson *et al.*, 1993; 1996). SPSS was used to generate tables with numbers of subjects with each value of gene dosage, for the different subsets of subjects within a study (*e.g.* individuals with or without tardive dyskinesia, or TD). From these tables, the data were collapsed into 2x2 contingency tables for entry into EpiInfo, as follows:

	With Disease (*e.g.* With TD)	Without Disease (*e.g.* Without TD)
With exposure (*e.g.* CYP2D6 PM status)		
Without exposure (*e.g.* CYP2D6 non-PMs)		

These tables were then analysed (using EpiInfo) by χ^2, or, if the number in individual cells was less than 5, by Fisher's exact test. EpiInfo also generates odds ratios (ORs), with 95% confidence limits (Cornfield or exact) of these.

For the genotyping study involving *CYP2C19* (tricyclic antidepressant, or TCA response), the number of functional *CYP2C19* genes was calculated similarly, except that for this locus, there are not known gene duplication events, so the number of functional genes varies between 0 and 2 (0 = homozygous for non-functional allele, 1 = heterozgous for non-functional allele, 2 = homozygous wild-type). For EpiInfo, analysis was conducted using the recessive model, appropriate here for a gene coding a metabolic enzyme.

With the genotyping study involving the $T_{-3591}G$ CYP1A2 promoter mutation, the functional effect of this mutation is not known. The genotypic distribution in individuals of different ethnic groups was calculated, and χ^2 analysis using EpiInfo was conducted comparing homozygous mutant versus all others (recessive model), or heterozygous plus homozygous mutant versus wild-type (dominant model).

In all the genotyping studies, the genotypic distribution was tested for deviation from Hardy-Weinberg equilibrium using the χ^2 test using the programme HWE (Utility programs for analysis of genetic linkage, J. Ott, 1999). If a population with a bi-allelic system with two alleles (A and a) of frequencies p and q respectively is in Hardy-Weinberg equilibrium, the frequencies of each genotype (AA, Aa, and aa) are p^2, $2pq$, and q^2, respectively, and remain stable over successive generations. However, the Hardy-Weinberg equilibrium holds exactly only for large populations in which there is random mating and in which there is no selection, mutation, or migration. Deviation from any of these conditions can alter allele frequency in a population and lead to an increase or decrease in allele frequencies from one generation to another (Gelehrter *et al.*, 1998).

2.3.2.2 Allelic distribution

Similarly, SPSS was used to generate the frequencies of the allelic variants identified in the sample groups tested, and the numbers of subjects of each type of allelic variant (in the case of CYP2D6, wild-type, versus non-functional, versus functional gene duplication) were generated from the genotypic tabulation. From such tables, 2x2 contingency tables were derived, and EpiInfo was used to perform a χ^2 or Fisher's exact test as appropriate, with OR and 95% confidence limits, with the exposure categories being those subjects with and without the allelic variant type of interest (*e.g.* non-functional allele).

2.3.2.3 Testing for interactional effects

For the TCA response study, the combined effect of CYP2D6 and CYP2C19 activity was analysed by two different ways:

1 A simple model, assuming additive effect of number of CYP2D6 and CYP2C19 genes: creating a new variable by multiplying the number of *CYP2D6* and number of *CYP2C19* functional genes together, and analysing for association with clinical response, *etc.*

2 The creation of a variable (combined CYP2D6 and CYP2C19 index) approximating to the combined effect of these CYP2D6 and CYP2C19 activities as reflected in the serum level data: (the inverse of the dose corrected combined TCA level) x (the demethylation index), see Section 3.3.2.

2.3.2.4 Bonferroni correction

A Bonferroni adjustment was applied where there was multiple testing, *i.e.* α, the significance level, was set as 0.05 divided by the number of independent tests performed (Bland & Altman, 1995).

2.3.3 Regression analysis

2.3.3.1 Logistic regression

Logistic regression with a dichotomised variable as the dependent variable (*e.g.* dichotomised response to TCA, responders being defined as those with a Hamilton Depression Rating Scale score of 15 or less after at least 6 weeks of treatment with TCA) was conducted using SPSS, controlling for independent confounding variables, with the variable of interest (*e.g.* number of functional *CYP2D6* genes) as the main independent variable of interest. The output including the regression coefficient, signficance value, and Wald statistic (the ratio of the square of the regression coefficient to the square of the standard deviation; the Wald statistic is calculated by the programme for the variables in the model to determine whether or not a variable should be removed), was then tabulated.

2.3.3.2 Multiple regression

Multiple linear regression was similarly conducted using SPSS, using a linear variable (*e.g.* percentage change in Hamilton Depression Rating Scale score) as the dependent variable, controlling for independent confounding variables (*e.g.* other concomitant medications that inhibit CYP2D6), with the main variable of interest (*e.g. CYP2D6* gene dosage) being again the main independent variable.

2.3.4 Estimation of sample power

Estimations of sample power were calculated using Solo Power Analysis and NCSS.

2.3.4.1 Power of Chi-squared test

The power of the Chi-squared test for a given sample was calculated using Solo Power Analysis, inputting the sample size, appropriate degrees of freedom, and different effect sizes. For example, the sample of 246 clozapine-treated patients has a power of 1.0 (allelic or genotypic comparison, one degree of freedom) at the $P < 0.05$ level to detect a medium effect size ($w = 0.3$) for the χ^2 test between the intolerant and non-intolerant groups (Cohen, 1977). With a smaller effect size ($w = 0.2$), the power remains high at 0.99 (allelic) or 0.88 (genotypic).

2.3.4.2 Power of logistic regression analysis

The power of logistic regression analysis of a given sample was calculated using NCSS, entering values for the relevant variables based on the study results. For example, for the study on TD, p0 = base proportion, *i.e.* fraction of the sample affected, equal to 0.18 of the sample of 72 rated for probable TD, α, or the significance level, was set at 0.05, the odds ratio was 4.86, and R^2 was 0.0469 (R^2 being calculated by linear regression of gene dosage onto the other variables), giving a power of 0.999.

2.3.5 Analysis of transfection data

The transfection data were analysed using Instat Biostatistics (Graph Pad, Intuitive Software for Science, San Diego, CA, USA), and SigmaStat 2.0 (SPSS Inc., Chicago, IL., USA). A Bartlett's test for homogeneity of variances was initially performed,

which indicated that non-parametric analysis should be used. The Kruskal-Wallis test was used, with a post-hoc Dunn's Multiple Comparisons Test to compare the results of each of the groups of transfections versus the wild-type. The mean fold induction for each transfection experiment performed for the 4 plasmids was similarly analysed using the Kruskal-Wallis test.

2.3.6 Analysis of pharmacokinetic data

Pharmacokinetic data were calculated using the equation:

$$Cl = D \times F / AUC$$

in which Cl is whole blood clearance, D is doses of clozapine, AUC is the area under the curve, and F is the fraction absorbed or bioavailability (model independent pharmacokinetics). The AUC was calculated using the linear trapezoidal rule using C = 0 and t = 0 and was extrapolated to infinity using Axum 5.0. The half-life of clozapine in blood was calculated by linear regression of the ln transformed blood concentrations. The percentage of the clozapine clearance mediated by CYP1A2 ($\%Cl_{CYP1A2}$) in wild-type mice was estimated by the following relationship:

$$\%Cl_{CYP1A2} = (Cl_{w\text{-}t} - Cl_{-/-})/Cl_{w\text{-}t}$$

in which $Cl_{w\text{-}t}$ is the wild-type clozapine clearance and $Cl_{-/-}$ is the clozapine clearance in the CYP1A2 -/- mice. This simple relationship was used as it had already been established that the CYP1A2 -/- and wild-type mice lines did not differ from each other

in parameters (such as liver function) that could affect the pharmacokinetics of clozapine (Buters *et al.*, 1996).

Statistical analyses comparing the parameters in the wild-type versus the CYP1A2 -/- mice were conducted using SPSS. Although all the parameters measured would be expected to show a normal distribution, non-parametric testing (the Mann-Whitney *U*-test) was chosen because there were only 4 mice per group, and hence non-parametric testing was more appropriate and more stringent.

CHAPTER THREE

PHARMACOGENETIC ASSOCIATION STUDIES

3.1 CYP2D6 Genotype-phenotype correlation studies

3.1.1 Introduction

3.1.1.1 Previous CYP2D6 genotype-phenotype correlation studies

Early CYP2D6 genotype-phenotype correlation studies include those of Daly *et al.* (1991) and Broly *et al.* (1991). These studies used phenotyping with debrisoquine and genotyping using Southern blotting with restriction fragment length polymorphism analysis (RFLP) as well as PCR-based methods for detecting the *CYP2D6*3, CYP2D6*4, CYP2D6*5,* and in the latter case, *CYP2D6*9* variants (then known as *CYP2D6A*, *CYP2D6B*, *CYP2D6D*, and *CYP2D6C* respectively). Using these techniques, Broly *et al.* reported identification of at least 95% of mutant poor metabolising alleles: 96.4% of individuals were correctly predicted, 100% of the extensive metabolisers, and 86% of the poor metabolisers. A similar study by Graf *et al.* (1992) used only allele-specific PCR amplification (methodology of Heim & Meyer, 1990) for *CYP2D6*3*, *CYP2D6*4*, and *CYP2D6*9*, and reported 97.5% correct classification of EMs, and 75% correct classification of PMs (with no *CYP2D6*9* alleles being detected). Studies in Black Africans revealed a dissociation between debrisoquine, sparteine, and metoprolol metabolic ratios (Lennard *et al.*, 1992; Masimirembwa *et al.*, 1996a), and a higher median value for the metabolic ratio as compared with Caucasians, which was later found to be due to the presence of the

*CYP2D6*17* allele in this ethnic group (Masimirembwa *et al.*, 1996b). The relatively high frequency of the *CYP2D6*17* allele in individuals of African origin was confirmed in a detailed analysis of debrisoquine phenotype-CYP2D6 genotype in American Caucasians and African-Americans reported by Daly and colleagues (Leathart *et al.*, 1998).

In recent years, many different *CYP2D6* alleles have been identified, with the result that there are now (http://www.imm.ki.se/CYPalleles, searched 23rd July 2002, page updated by Mikael Oscarson 3rd July 2002) a total of 43 identified *CYP2D6* alleles, 17 major alleles (with subtypes) identified as having complete lack of activity, 7 alleles with reduced activity, and several allelic variants for which the functional consequence has not yet been defined (see Section 1.5.1.1). These variants include SNPs, deletions, and *CYP2D67P/CYP2D6* hybrid alleles produced by unequal crossover events (the latter including *CYP2D6*13* and *CYP2D6*16*, Daly *et al.*, 1996b). In addition, duplications or amplifications of *CYP2D6*1*, *CYP2D6*2*, and *CYP2D6*4* have been described. The most up-to-date information on CYP2D6 alleles can be found on the website.

A landmark genotyping-phenotyping correlation study was that of Sachse *et al.* (1997), in which 589 unrelated German volunteers were phenotyped with either dextromethorphan (456) or debrisoquine (133), and genotyped for *CYP2D6* alleles **1-*16*, as well as for gene duplication events, including discrimination of which allele was duplicated. Observed CYP2D6 genotypes were grouped into those denoting none, one, two, or three functional alleles, and significant differences ($P < 0.001$) were demonstrated in mean dextromethorphan MR between carriers of 3 (mean MR =

0.002), two (mean MR = 0.003), one (mean MR = 0.009), and no functional alleles (mean MR = 1.902), and between carriers of one versus two ($P < 0.001$) or three ($P < 0.01$) functional alleles.

In another study by Marez *et al.* (1997), several novel variants were characterised by single strand conformation polymorphism (SSCP) analysis, but as noted by Sachse and colleagues (1997, 1998), most of the variants were very rare, and determination of the CYP2D6 alleles lacking in activity (**3-*6*) that are common in Caucasians, together with assays for *CYP2D6* gene duplication should be sufficient for a highly reliable prediction of CYP2D6 phenotype amongst the EM, IM, and PM categories.

There has been one other large phenotyping study of French Caucasians (Laforest *et al.*, 2000, performed after I commenced my study). This was a CYP2D6 genotype-phenotype study in lung cancer patients and hospital controls, genotyping for *CYP2D6*3-5* and **16*, and gene amplification events, and phenotyping with dextromethorphan. In this study, genotypes corresponding to UM, EM, HEM (heterozygous EM/PM), and PM status were detected in 4.7%, 62.6%, 25.3%, and 6.4% of the controls. The *CYP2D6*4* allele frequency in the controls was 0.149 (allele frequencies of the other alleles not given). There was 93.8% concordance (121/129 controls) between the genotypic and phenotypic determinations, with two genotypic PMs for whom the dextromethorphan MR was below 0.3, and two genotypic EMs that were phenotypically determined as PMs. Phenotypically, the frequency of PMs was 8.5%.

3.1.1.2 Aims of these studies

My French Caucasian sample consisted of 154 male general population volunteers, prior to participation in a Phase II Clinical Trial.

The aims of the study were:

1 To genotype the volunteers, in order to identify individuals homozygous for non-functional alleles (PMs), and individuals homozygous wild-type (EMs), so that these individuals could participate in the Phase II trial.

2 To investigate the degree of genotype-phenotype correlation in the sample, through genotyping for the *CYP2D6*3-5* and gene duplication alleles. At the time of the study, there were no genotype-phenotype studies in French Caucasians.

3 To use the long-PCR assay that I developed to identify the frequency of individuals positive for a CYP2D6 duplication/amplification event in the 154 male volunteers, and to compare the results of this assay with that of the long-PCR assay for CYP2D6 gene duplication developed by Løvlie *et al.*, 1996.

The UK Caucasian sample was recruited by the Clinical Age Research Unit, King's College London, and consisted largely of volunteers of at least 60 years of age. The aim of this study was to investigate the degree of CYP2D6 genotype-phenotype correlation in an elderly sample, in order to ascertain whether due to age, the CYP2D6 metabolising capacity (and hence phenotype) was reduced, and hence a relative genotype-phenotype discrepancy developed.

3.1.2 French sample

3.1.2.1 Methods

One hundred and fifty-four male French volunteers taking no prescribed medication were enlisted for the study (Section 2.1.1.1). Of these, one hundred and thirty-six were Caucasian, 17 of North African descent, and 1 was Black African in origin. The mean age was 26.15 years (sd 4.15, range 19-39 years).

DNA was extracted from blood collected in EDTA tubes using the Nucleon II kit (now marketed as the Nucleon BACC3 kit, see Section 2.2.2.1), and genotyping for *CYP2D6*3-5* and gene amplification events undertaken. A subset of this sample, consenting to phenotyping and with no contraindications (N = 46) were phenotyped with dextromethorphan (Section 2.2.1.2).

*CYP2D6*4* and *CYP2D6*5* are the most common and next most common null alleles respectively; analysis for *CYP2D6*4*, *CYP2D6*5*, and *CYP2D6*3* would be predicted to detect 90 to 95% of null alleles in a European Caucasian population (Heim *et al.*, 1990; Broly *et al.*, 1991; Dahl *et al.*, 1992). The *CYP2D6*3* and *CYP2D6*4* point mutation alleles were detected by PCR followed by restriction enzyme digestion as in the method of Smith *et al.*, 1992, with minor modifications. For the *CYP2D6*3* assay, primers 5'-ATGAGCTGCTAACTGAGCCC-3' and 5'-CCGAGAGCATACTCGGGAC-3' were used in a total reaction volume of 25 µl with the buffer typical for standard length PCR amplification (Section 2.2.3.1), with 3 mM $MgCl_2$, 0.2 mM each dNTP, 0.25 µM each primer, and 1.25U AmpliTaq (Perkin-Elmer, UK). Cycling conditions were: initial denaturation at 94^0C for 3 min, 30 cycles

at 95^{0}C for 1 min, 60^{0}C for 30 s, and 72^{0}C for 1 min, followed by final elongation at 72^{0}C for 10 min. PCR products were digested using *Hpa* II, and analysed on a 3% agarose gel, together with a 1kb ladder (Gibco BRL). For the *CYP2D6*4* assay, I used primers 5'-GCCTTCGCCAACCACTCCG-3' and 5'-AAATCCTGCTCTTCCGAGGC-3' and the same reaction conditions and cycling conditions as for *CYP2D6*3*, except that a $MgCl_2$ concentration of 1.5 mM was used. PCR products were digested with *Bst* NI and analysed on a 3% agarose gel. The *CYP2D6*5* gene deletion allele was assayed by long-PCR using the GeneAmp XL PCR kit (Perkin Elmer, UK) by the method of Steen and colleagues (1995), as described in Section 2.2.3.1. Figures 3.1, 3.2, and 3.3 show representative results of assays for *CYP2D6*3*, *CYP2D6*4*, and *CYP2D6*5*.

CYP2D6 gene amplification was detected by the long-PCR method of Løvlie *et al.*, 1996 (Section 2.2.3.2), and for the forty-six cases that were phenotyped, the long-PCR assay that I developed for CYP2D6 gene amplification was also used (Section 2.2.3.2).

The results were analysed using SPSS for Windows, EpiInfo Version 6 and Axum 5.0 (Section 2.3).

3.1.2.2 Results

Nine out of 154 subjects (5.8%) were genotyped as PMs (having no functional *CYP2D6* alleles). The relationship between dextromethorphan metabolic ratio and *CYP2D6* genotype for the 46 individuals that were phenotyped is shown in Figure 3.1 (antimode = 0.3, *i.e.* MR > 0.3 defining PMs, and MR ≤ 0.3 defining EMs and UMs). The mean dextromethorphan MR for the forty-six individuals was 0.482 (sd 1.97), and

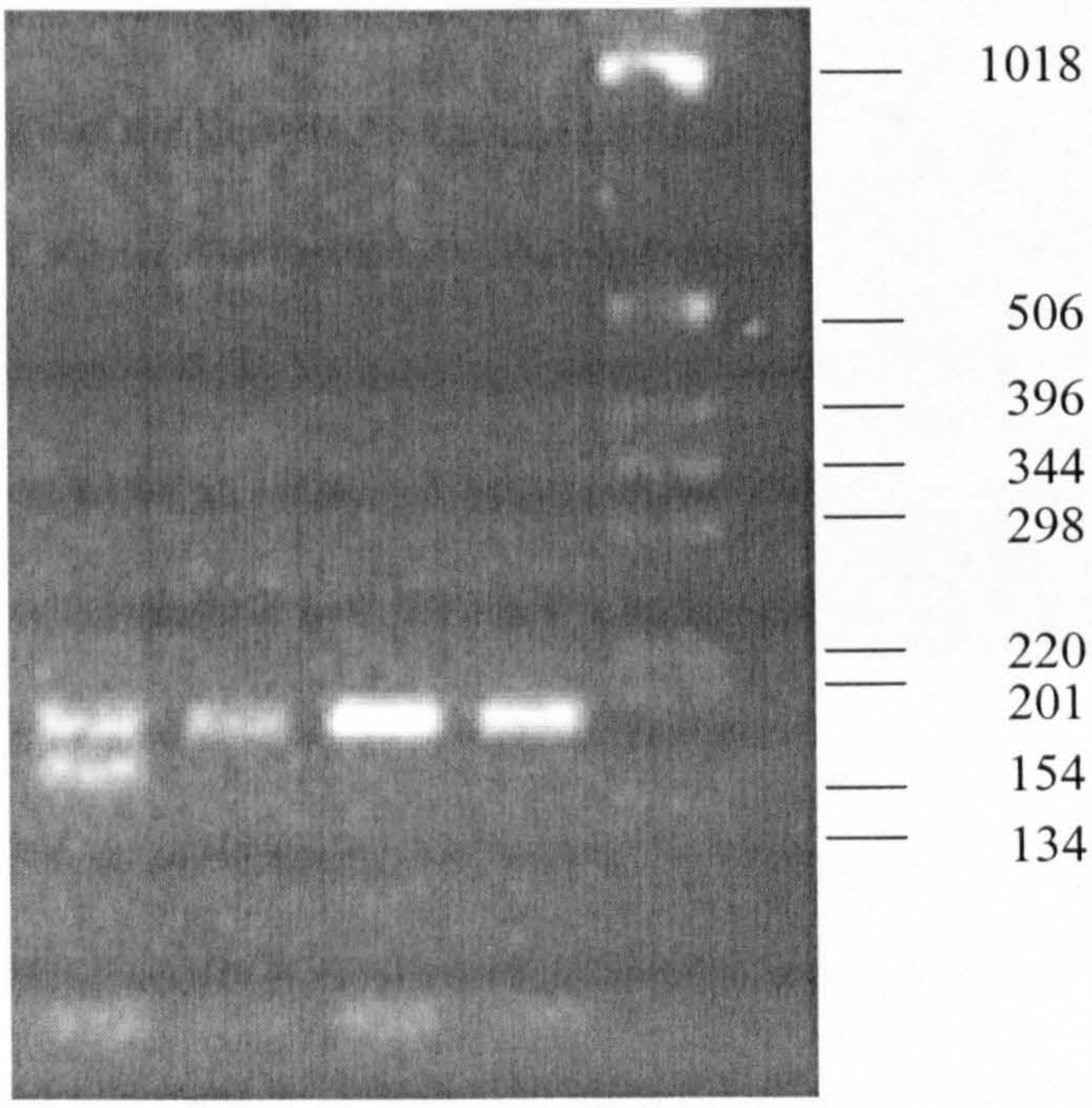


Figure 3.1 Agarose gel electrophoresis of *Hpa* II digests for *CYP2D6*3* analysis. The subject in lane 1 is heterozygous for the *CYP2D6*3* allele, the subjects in lanes 2-4 are wild-type, and in lane 5 is a 1 kb ladder (Gibco BRL, Inc., bp).

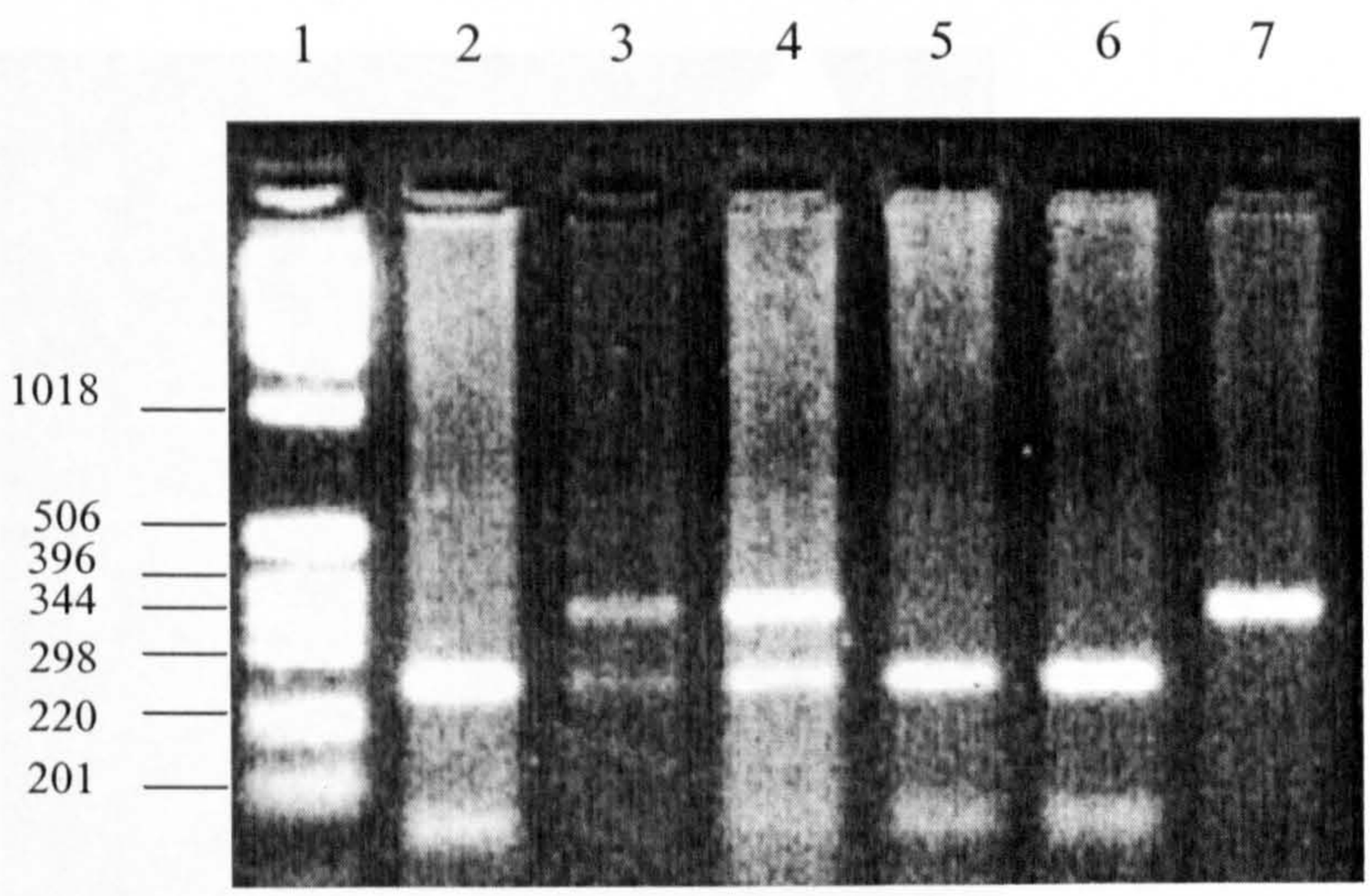


Figure 3.2 Agarose gel electrophoresis of *Bst* NI digests for *CYP2D6*4* analysis. Subjects in lanes 2, 5, and 6 are wild-type, subjects in lanes 3 and 4 are heterozygous for *CYP2D6*4*, and the subject in lane 7 is homozygous for *CYP2D6*4*. In lane 1 is a 1 kb ladder (Gibco BRL, Inc., bp).

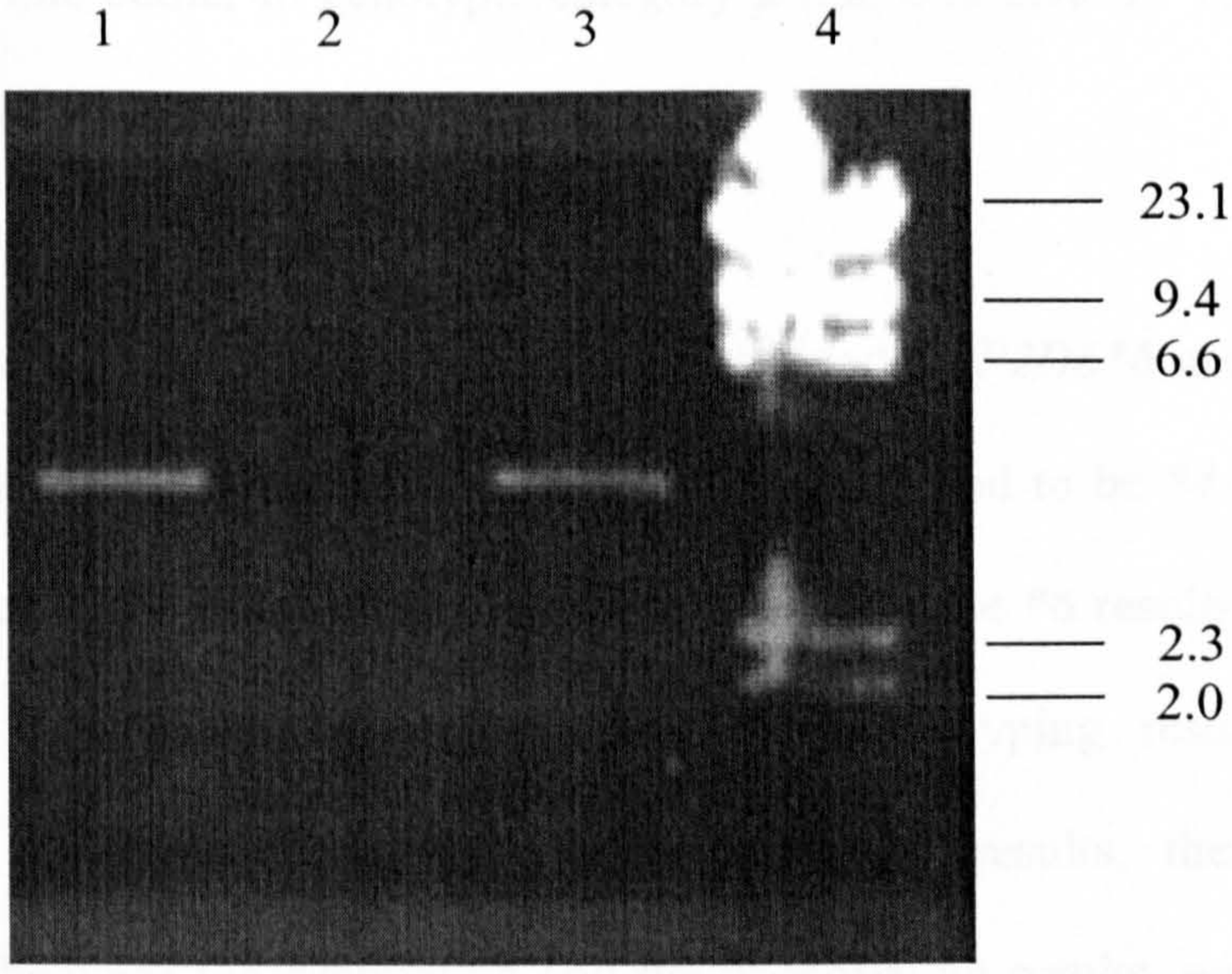


Figure 3.3 Agarose gel electrophoresis of long-PCR products for *CYP2D6*5* analysis. The subject in lane 1 is positive for *CYP2D6*5*, in lane 3 is a positive control (supplied by Dr A Daly), and in lane 4 is a λ/*Hind* III ladder (Gibco BRL, Inc., kb).

for the Caucasian subset (N = 44) was 0.483 (sd 1.97). It is clear in Figure 3.4 that two out of 46 cases were genotypically EMs (heterozygous EMs), but phenotypically PMs. There is also one outlier in genotypic category 2 (*i.e. CYP2D6*1/*1*), with an MR of 0.070 ($\log_{10}$MR = -1.15).

These two cases were genotyped using the combined *CYP2D6*6* and **4* allele assay (methodology described by Sachse *et al.*, 1997), and found to be **4/*6* genotype. No cases were genotypic PMs and phenotypic EMs. With the **6* results there is a 100% concordance between the genotyping and the phenotyping results in terms of classification of PMs (Figure 3.5). Without the **6* results, there was 95.6 % concordance between the genotyping and the phenotyping results, with the sensitivity of the genotyping being 100% and the specificity 95.2% (calculated with the phenotyping result representing the "actual" condition, and the genotyping result the "test" condition). Interestingly, Laforest *et al.* (2000) did not genotype for *CYP2D6*6*, and had only 93.8% concordance between their genotyping and phenotyping results. This suggests that it is necessary to genotype for *CYP2D6*6* in order to identify all PMs in French Caucasians.

Table 3.1 gives the numbers and percentages of cases detected in the Caucasians from my study with 0, 1, 2, or 3 functional genes, in comparison to the data of Sachse *et al.* (1997), Leathart *et al.* (1998), and Laforest *et al.* (2000). The percentages of cases in my study are seen to be similar to those of both of these two groups of investigators, and to lie within the 95% confidence limits given by Sachse *et al.* (1997). Moreover, statistical comparison using the χ^2 test (3 degrees of freedom), between my data and the raw data of these other investigators reveals that my data do not differ significantly in

	French[1]	British[2]	British >60 years[3]	American[4]	French[5]	German[6]	
Number of functional CYP2D6 genes	N = 136	N = 72	N = 65	N = 247	N = 171	N = 456	95% CL
0	10 (7.4)	5 (6.9)	2 (3.1)	18 (7.3)	11 (6.4)	33 (7.24)	5.03 – 10.0
1	43 (31.6)	29 (40.3)	27 (41.5)	69 (27.9)	45 (26.3)	165 (36.2)	31.8 – 40.8
2	77 (56.6)	35 (48.6)	33 (50.8)	154 (62.3)	107 (62.6)	246 (53.9)	49.2 – 58.6
3	6 (4.4)	3 (4.2)	3 (4.6)	6 (2.4)	8 (4.7)	12 (2.63)	1.37 – 4.55

Table 3.1 Distribution of numbers of functional *CYP2D6* genes in French, British, British elderly, American, and German Caucasians (numbers with percentages in parentheses given in each group); [1]French Caucasians (Section 3.1.2), [2]UK Caucasians (Section 3.1.3), [3]elderly UK Caucasians (age > 60 years, section 3.1.3), [4]American Caucasians reported by Leathart *et al.* (1998), [5]Caucasians from French sample of Laforest *et al.* (2000), [5]German Caucasians reported by Sachse *et al.* (1997), with 95% confidence limits for this sample

distribution from that of Sachse *et al.* ($\chi^2 = 1.85$, $P = 0.60$), or Leathart *et al.* ($\chi^2 = 1.99$, $P = 0.58$), or Laforest *et al.* ($\chi^2 = 1.30$, $P = 0.73$).

The mean dextromethorphan MRs for the different genotypic categories and for subjects with 0, 1, or 2 functional (active) *CYP2D6* genes is given in Table 3.2, with comparative data from Sachse *et al.* (1997). (There were no individuals with 3 functional genes amongst the forty-six who were phenotyped.) If the outlier in genotypic category 2 is excluded, then the mean MR for subjects with 2 functional copies of CYP2D6 is the same as those in the study reported by Sachse *et al.* (1997). The mean MR for subjects with 1 functional *CYP2D6* gene in my study (0.028) is higher than that for the same group of subjects in Sachse *et al.* (1997), *i.e.* 0.009, and is outside the 95% confidence limits reported by Sachse *et al.* (0.007-0.012).

The frequencies of *CYP2D6* alleles **3-5* and gene amplification in the whole sample, and in the Caucasian and African subsets are shown in Table 3.3. The genotypic distributions were in Hardy-Weinberg equilibrium for all the *CYP2D6* variants tested, in the whole sample, and Caucasian and African subsets. The allele frequencies of the Caucasians in my sample are seen to be similar to the results of Sachse *et al.* (1997), while the subjects of African descent have a lower frequency of *CYP2D6*3* (no alleles detected) and *CYP2D6*4*, and a higher frequency of the gene amplification event (4 out of the total of 18, i.e. 22.2%, of non-Caucasian cases were all **1/*1* genotype and positive for the gene amplification). The allele frequency of the **6* allele is not given for my French sample as only the two individuals with genotype-phenotype discrepancy were genotyped for **6*. The allele frequency of *CYP2D6*6* in the 589

	French males[1]		German Caucasians[2]	
***CYP2D6* genotype**	N	Mean MR (sd)	N	Mean MR (sd)
**1x2/*4*	1	0.002		
**1/*1*	26	0.006 (0.013)		
Σ2 functional genes	27	0.006 (0.013)	246	0.003 (0.002 – 0.003)
Σ2 functional genes, excluding outlier	26	0.003 (0.003)		
**1/*3*	1	0.050		
**1/*4*	11	0.02 (0.031)		
**4x2/*1*	1	0.090		
Σ1 functional gene	13	0.028 (0.035)	165	0.009 (0.007 – 0.012)
**3/*4*	2	2.075 (0.163)		
**4/*4*	2	1.225 (0.728)		
**6/*4*	2	7.08 (8.09)		
Σ0 functional genes	6	3.46 (4.60)	33	1.902 (1.392 – 2.598)

Table 3.2 Dextromethorphan MRs (metabolic ratios) corresponding to different genotypic groups, for forty-six French male volunteers (section 3.1.2),[1] and four hundred and forty-four German Caucasians reported by Sachse *et al.* (1997)[2]

Sample details	*CYP2D6*3*	*CYP2D6*4*	*CYP2D6*5*	Duplication allele
French males (all ethnicities) (N = 154)[1]	0.009	0.173	0.033	0.042
French Caucasians (N = 136)[1]	0.011	0.174	0.033	0.033
French individuals of African origin (N = 18)[1]	0	0.111	0.028	0.111
UK Caucasians[2]	0.007	0.246	0.035	0.021
UK elderly Caucasians (> 60 years) (N = 65)[3]	0	0.208	0.031	0.021
German Caucasians (N = 589)[4]	0.020	0.207	0.0195	0.0193
(with 95% CL)	(0.0131-0.0302)	(0.184-0.231)	(0.0124 – 0.0292)	(0.0097 – 0.060)
African Americans[5]	0.006	0.073	0.069	0.024

Table 3.3 *CYP2D6* allele frequencies in [1]French male volunteers of varying ethnicity (Section 3.1.2), [2]UK Caucasians (Section 3.1.3), [3]elderly UK Caucasians (age > 60 years, section 3.1.3), [4]German Caucasians reported by Sachse *et al.* (1997), and [5]African-Americans reported by Leathart *et al.* (2000)

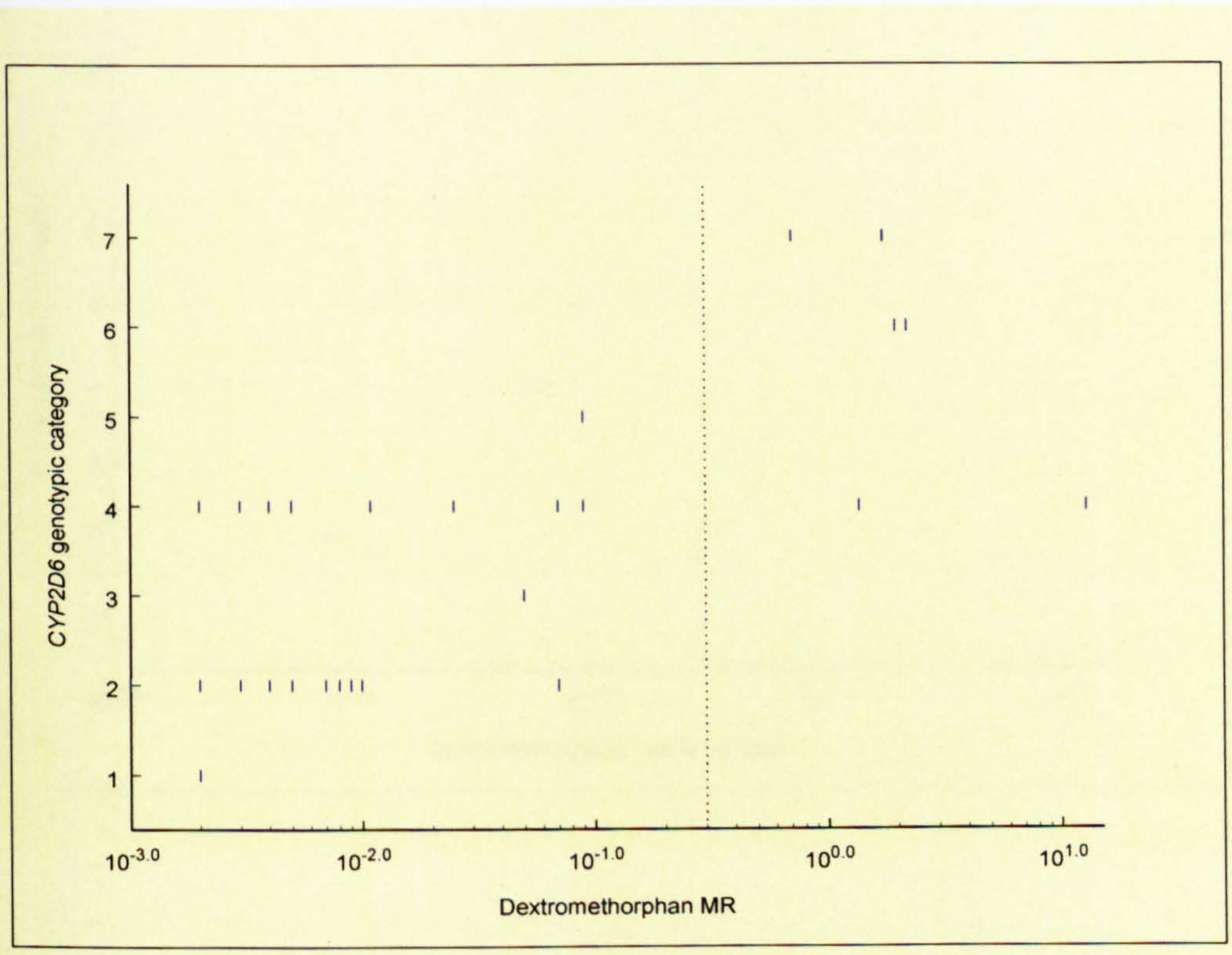


Figure 3.4 CYP2D6 phenotype (dextromethorphan metabolic ratio) versus *CYP2D6* genotypic category for 46 French male volunteers, without the **6* data (where 1 = **1x2/*4*, 2 = **1/*1*, 3 = **1/*3*, 4 = **1/*4*, 5 = **4x2/*1*, 6 = **3/*4*, and 7 = **4/*4*). The dotted line marks a metabolic ratio of 0.3 (antimode, MRs greater than 0.3 phenotypically defining poor metabolisers).

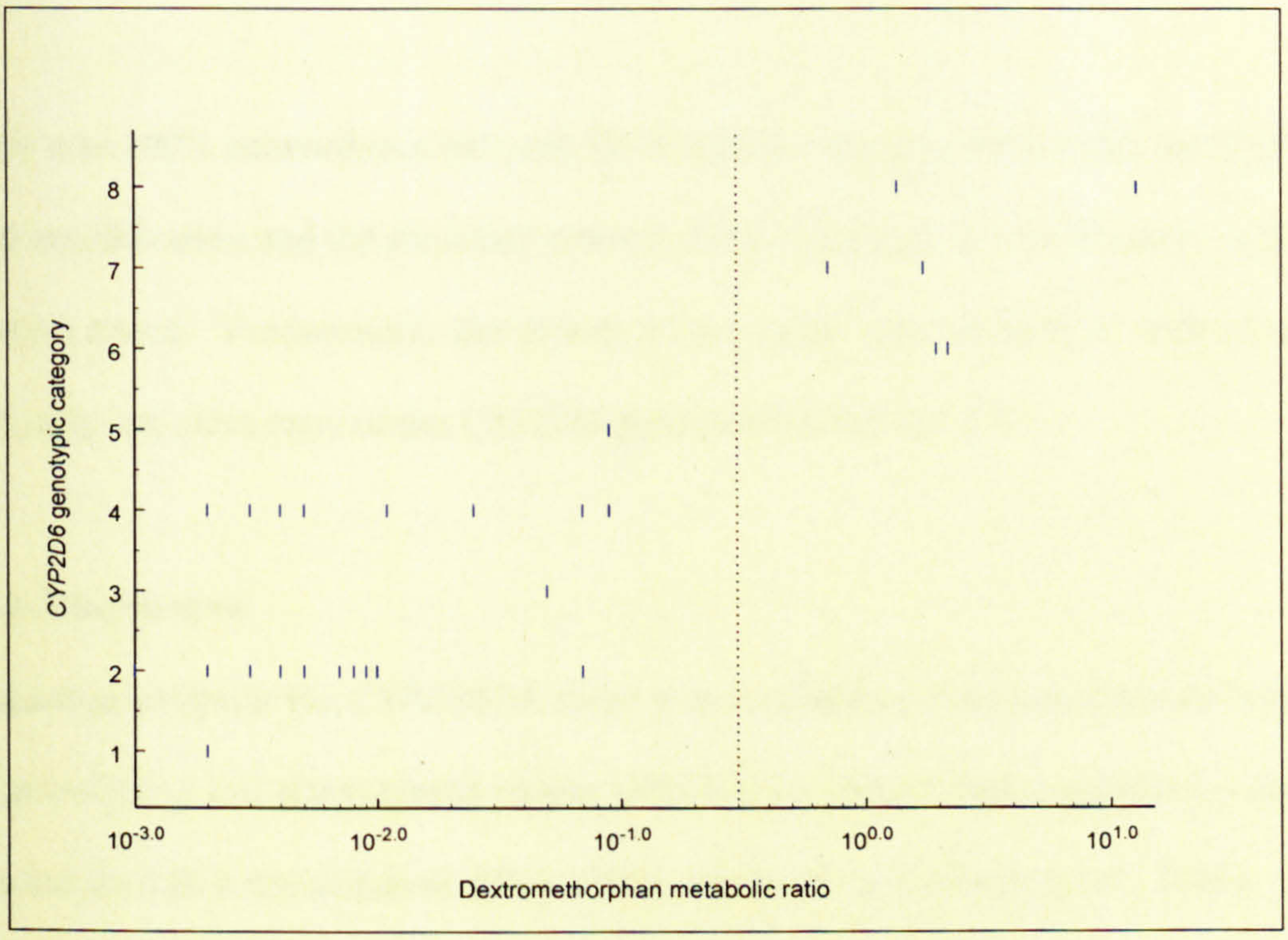


Figure 3.5 CYP2D6 phenotype (dextromethorphan metabolic ratio) versus *CYP2D6* genotypic category for 46 French male volunteers, with the **6* data (where 1 = **1x2/*4*, 2 = **1/*1*, 3 = **1/*3*, 4 = **1/*4*, 5 = **4x2/*1*, 6 = **3/*4*, and 7 = **4/*4*, and 8 = **4/*6*). The dotted line marks a metabolic ratio of 0.3 (antimode, MRs greater than 0.3 phenotypically defining poor metabolisers).

in the 589 German Caucasians reported by Sachse *et al.* (1997) was 0.0093.

There was 100% concordance between the results for my long-PCR assay for CYP2D6 gene amplification and the modified version of the Løvlie *et al.* (1996) assay in the 46 samples tested. Furthermore, the results of my assay were consistent with an allele with only one extra copy of the *CYP2D6* gene (see Section 2.2.3.2).

3.1.2.3 Discussion

Without genotyping for *CYP2D6*6*, there was a relatively high concordance between the genotyping and phenotyping results (95.6%), consistent with concordance figures reported by other investigators (93.4-100%, reviewed in Laforest *et al.*, 2000). With the **6* results (together with **3-5* and gene amplification analysis), the concordance was 100%. The percentages of cases with 0,1, 2, or 3 functional genes was not significantly different from those in the studies of Sachse *et al.* (1997), Leathart *et al.* (2000), or Laforest *et al.* (2000). The allele frequencies in the Caucasians of my sample were also similar to those of Sachse *et al.* (1997), while the pattern of frequencies seen in the subjects of African descent was similar to those in the study of Leathart *et al.* (1998), with a lower frequency of *CYP2D6*3* and **4* in the subjects of African origin.

However, the mean dextromethorphan MR for the French Caucasians in this sample (0.483) was higher than the mean dextromethorphan MR for the German Caucasian sample reported by Sachse and colleagues (0.143), the mean MR for subjects with 1 functional gene (0.028) was also higher than that reported by Sachse *et al.* (0.009), and there was one outlier in terms of phenotype corresponding to **1/*1* genotype. The

increased overall mean dextromethorphan MR and increased mean MR for subjects with 1 functional *CYP2D6* gene may either be an artefactual finding due to the relatively small sample size, or represent an increased incidence of *CYP2D6* alleles that may be associated with reduced CYP2D6 activity (e.g. *CYP2D6*2*, which I did not assay for in this sample, and which may be associated with reduced *CYP2D6*2* activity; compare with the effect of the *CYP2D6*10* and **17* alleles on the mean MR in Orientals and individuals of African origin, section 1.5.1.1). Alternatively, to explain both this and the 1 outlier, there is the possibility that the volunteers were taking non-prescribed medications (e.g. cough remedies) that were CYP2D6 inhibitors. In the case of the **1/*1* outlier, although the MR value is outside the 95% confidence limits of the MR values for subjects with this genotype in the study of Sachse *et al.* (1997), it is not outside the range seen in that study. This individual is a Caucasian, and further genotyping of this subject is in progress.

Sachse *et al.* (1997) explored the functional effect of alleles *CYP2D6*2*, **9*, and **10* by comparison of the metabolic ratios corresponding to different allelic combinations, and found significant differences ($P < 0.001$) between PM/PM, and all other combinations, and between IM/PM and EM/EM, EM/IM, and IM/IM. Comparison of genotypes including the *CYP2D6*2* allele with those including the wild-type allele **1* showed a higher mean dextromethorphan MR comparing **2/*4* and **1/*4* ($P < 0.01$), and a trend for a higher mean MR comparing **2/*2* with **1/*1*. In the phenotyping, the antimode was 0.3 for the dextromethorphan and 12.6 for the debrisoquine test, and all 8 individuals phenotyped as debrisoquine PMs were genotyped as PMs. In the dextromethorphan group, one individual had an MR of 0.47, and was genotypically heterozygous, and individuals with the **2/*4* genotype had MRs non-significantly

higher than 0.3. This is consistent with the debrisoquine test discriminating better than the dextromethorphan between poor and intermediate metabolisers, as Sachse *et al.* (1998) note. In the latter study, 47 phenotypically intermediate metabolisers as defined by a dextromethorphan ratio greater than 0.1 were re-phenotyped with debrisoquine, and genotyped by *Xba* I RFLP analysis and PCR-RFLP tests for *CYP2D6* alleles **1-*16*. One **1/*2* carrier, and 10 carriers of only one functional CYP2D6 allele had a debrisoquine MR ranging from 1.50 – 15.3, with the one individual having an MR greater than 12.6 having a dextromethorphan MR less than 0.3 (0.12), and no additional mutations being found on sequencing. The remaining 36 out of 47 individuals all had a debrisoquine MR greater than 12.6 and had no active *CYP2D6* allele. However, of note (see section 3.1.3.3), the findings of Zanger et al. (2001) suggest heterogeneity within alleles positive for the characteristic *CYP2D6*2* R296C substitution in that alleles with the G variant of the -1584C>G promoter SNP may have relatively high activity, whereas only those positive for both the R296C and the C variant at the promoter site are intermediate metabolising in terms of activity.

There was 100% concordance between the results of my long-PCR assay for the detection of *CYP2D6* gene amplification, and the results of the Løvlie *et al.* (1996) assay with primers cyp-17 and cyp-32 (i.e. my assay and the Løvlie et al., 1996 assay gave the same results in terms of identifying individuals with gene amplification). This demonstrates the validity of my assay. Moreover, I have shown that the number of functional *CYP2D6* genes on the allele that has been amplified can be estimated (Figure 2.5). Given that individuals with 3, 4, 5, and 13 copies of the *CYP2D6* locus on one allele have been described (Johansson *et al.* 1996; Aklillu *et al.*, 1996), this assay may well be useful to differentiate such individuals, who might be expected to be

particularly refractory to treatment with drugs that were significantly metabolised by CYP2D6 (e.g. tricyclic antidepressants), or sensitive to drugs that were activated by CYP2D6 (e.g. codeine). It is possible that the assay developed by Løvlie and colleagues might also be able to estimate copy number; further work should include comparison of the results of both assays on individuals of *CYP2D6* gene copy numbers 2, 3, 4, 5, and 13.

The higher frequency of the gene amplification in the subset of subjects of African descent is consistent with the results of Aklillu *et al.* (1996), who reported that 29% of Ethiopians were UMs, but differs from the results of Leathart *et al.* (1998), who reported 12/246 (4.9%) of African-American individuals were heterozygous for a *CYP2D6* gene duplication allele. Of my French subjects of African descent, 17 were of North African origin, 1 was Black African. The North Africans might be more ethnically similar to the Ethiopians, and hence my results are consistent with the above. Further study is warranted in British and French individuals of African origin.

3.1.3 UK Caucasians

3.1.3.1 Methods

A sample of 74 UK volunteers were recruited by the Clinical Age Research Unit, King's College London (Section 2.1.1.2), 32 males and 42 females. Of these, 67 were of age at least 60 years (elderly), 65 of whom were Caucasian, 29 were male, and 38 female. The mean age for those above 60 years was 71.31 years (sd 5.74, range 61-84 years). Of the total sample of 74, two were non-Caucasian in ethnicity (Caribbean). Forty-three volunteers were eligible for and consented to debrisoquine phenotyping.

The methodology for genotyping and data analysis (Section 3.1.2.1.1) and phenotyping was as described (Section 2.2.1.1).

3.1.3.2 Results

The relationship between debrisoquine metabolic ratio and *CYP2D6* genotype is shown in Figure 3.6 (antimode = 12.6, *i.e.* MR > 12.6 defining PMs). The mean debrisoquine MR for the 67 elderly was 5.67 (sd 15.3, range 0-70, where a value of zero means that only the metabolite was detected); the mean debrisoquine MR in the study by Sachse *et al.* (1997) was somewhat lower, 3.54 (calculated from Figure 2B in the paper).

From Figure 3.6 it can be seen that one out of 43 cases was genotypically an EM (heterozygous EM, **1/*5*), but phenotypically a PM. This individual was genotyped as wild-type on the **3* and **4* assays (i.e. it was possible to generate amplicons from the *CYP2D6* locus), and therefore cannot be homozygous for a *CYP2D6*5* deletion allele. This one case of genotype-phenotype discrepancy is negative on assay for *CYP2D6*6* and is being subjected to mutation screening by Dr K Tandon under my supervision, using denaturing high performance liquid chromatography (O'Donovan *et al.*, 1998). No cases were genotypic PMs and phenotypic EMs. This gives an overall 97.7% concordance between the genotyping and the phenotyping results, with the sensitivity of the genotyping being 100% and the specificity 97.4% (calculated on the assumption that the phenotyping result represents the "actual" condition, as above).

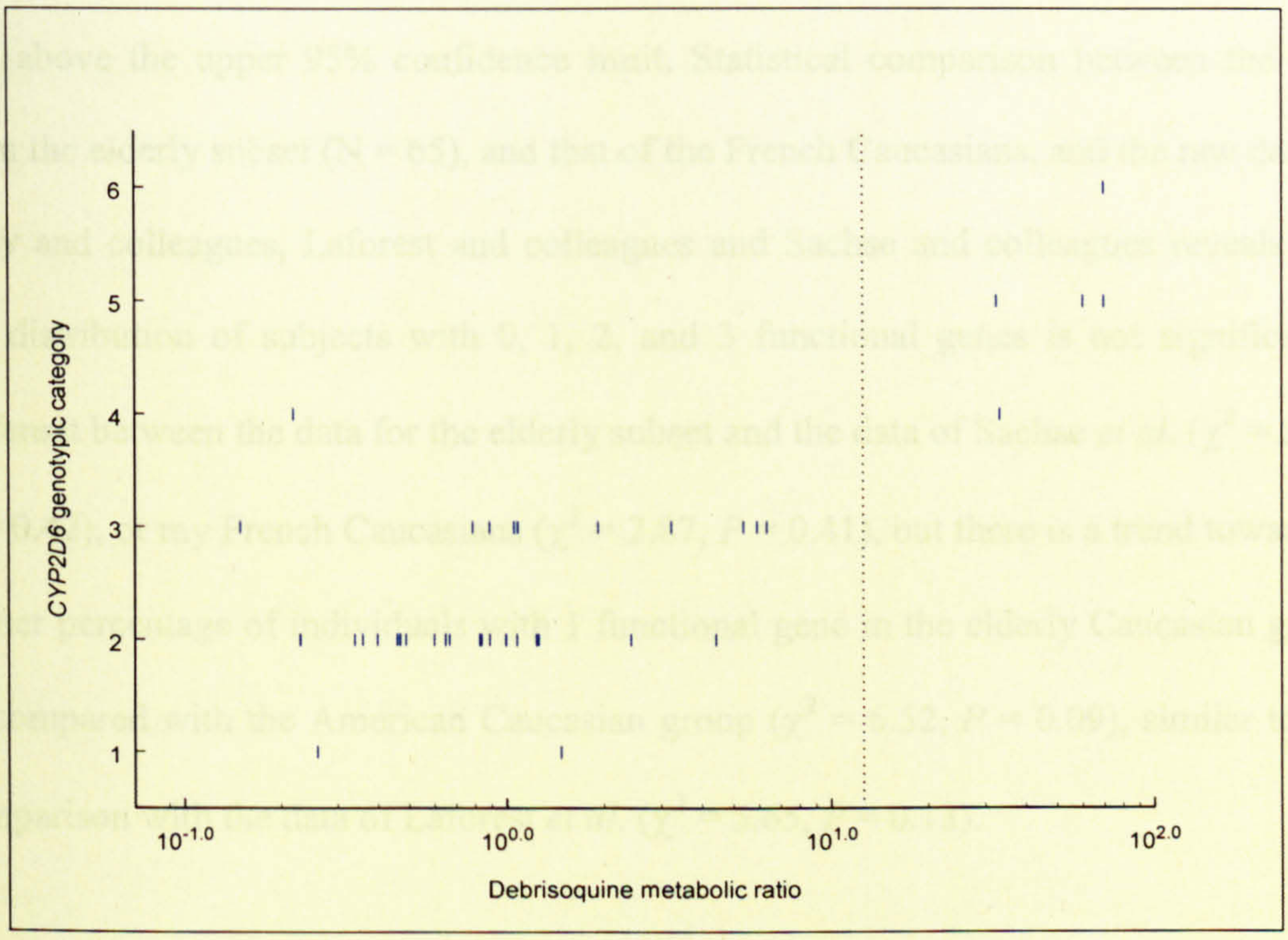


Figure 3.6 CYP2D6 phenotype (debrisoquine metabolic ratio) versus *CYP2D6* genotypic category for 40 UK volunteers (where 1 = **1x2/*1*, 2 = **1/*1*, 3 = **1/*4*, 4 = **1/*5*, 5 = **4/*4*, 6 = **4/*5*), metabolic ratios of zero excluded. The dotted line marks a metabolic ratio of 12.6 (antimode, MRs greater than 12.6 phenotypically defining poor metabolisers).

Table 3.1 gives the numbers and percentages of cases with 0, 1, 2, or 3 functional genes detected in the Caucasians from my study, and the elderly subset, in comparison to my data from the study on French subjects detailed above, and the data of Sachse *et al.* (1997), Leathart *et al.* (1998), and Laforest *et al.* (2000). The percentages of cases in the elderly subset lie within the 95% confidence limits given by Sachse *et al.* (1997),

except in the case of 1 functional gene, for which my percentage of cases detected is just above the upper 95% confidence limit. Statistical comparison between the data from the elderly subset (N = 65), and that of the French Caucasians, and the raw data of Daly and colleagues, Laforest and colleagues and Sachse and colleagues reveals that the distribution of subjects with 0, 1, 2, and 3 functional genes is not significantly different between the data for the elderly subset and the data of Sachse *et al.* ($\chi^2 = 2.79$, $P = 0.42$), or my French Caucasians ($\chi^2 = 2.87$, $P = 0.41$), but there is a trend towards a higher percentage of individuals with 1 functional gene in the elderly Caucasian group as compared with the American Caucasian group ($\chi^2 = 6.52$, $P = 0.09$), similar to the comparison with the data of Laforest *et al.* ($\chi^2 = 5.65$, $P = 0.13$).

The mean debrisoquine MRs for the different genotypic categories and for subjects with 0, 1, 2, or 3 functional (active) *CYP2D6* genes is given in Table 3.4, including the values if the outlier in genotypic category 4 is excluded. The corresponding values for debrisoquine MRs are derived from the data of Leathart *et al.* (1998), and it can be seen that my results fall within the ranges reported by Leathart and colleagues. Sachse *et al.* (1998) report a range for debrisoquine MR corresponding to no active *CYP2D6* genes of 17.1-249, for 1 functional gene 1.11-11.8, and the mean for 1 or 2 functional genes from the graphical presentation of the data in Sachse *et al.*, 1997 is approximately 1.44 and approximately 0.46 respectively. My results therefore also fall within the ranges reported by previous investigators, but the mean MR for subjects with one functional *CYP2D6* gene is higher in my study than in that of Sachse *et al.* or Daly and co-workers.

The frequencies of *CYP2D6* alleles **3-5* and gene amplification in the whole sample,

	UK Caucasians[1]		American Caucasians[2]	
***CYP2D6* genotype**	N	Mean (sd)	N	Mean (range)
**1x2/*1* (3 gene copies)	3	0.58 (0.79)	6[a]	0.54 (0.14 – 14.8)
**1/*1* (2 gene copies)	21	1.10 (1.22)	154[b]	0.54 (0.02 – 51)
**1/*4*	11	2.45 (2.40)		
**1/*5*	2	16.82 (23.47)		
Σ1 functional gene	13	4.66 (8.93)	69	1.55 (0.24 – 20.36)
Σ1 functional gene, excluding one outlier	12	2.26 (2.38)		
**4/*4*	4	58.24 (17.65)		
**4/*5*	1	70.00		
Σ0 functional genes	5	60.59 (16.16)	18	48.91 (5.45 – 244)

Table 3.4 Debrisoquine metabolic ratios corresponding to different genotypic categories, for UK Caucasians (section 3.1.2),[1] and American Caucasians reported by Leathart *et al.* (1998).[2] [a]Subjects with 3 gene copies, allelic combination not given, [b]subjects with 2 gene copies, allelic combination not given; means calculated from ln values reported

and in the elderly subset are shown in Table 3.3. The allele frequencies of the Caucasians in my sample are seen to be similar to the results for the French and German Caucasians, except that in the elderly UK Caucasians no *CYP2D6*3* was detected. The genotypic distributions for *CYP2D6* alleles *3-*5, and the gene duplication allele were all in Hardy-Weinberg equilibrium.

3.1.3.3 Discussion

In this study, there was 97.7% concordance between the genotyping and the phenotyping results. This is consistent with the findings of previous investigators, which have indicated that both phenotyping and genotyping may not be 100% accurate in the identification of true PMs (Griese *et al.*, 1998; Leathart *et al.*, 1998).

The mean debrisoquine MR (5.67) in the elderly sample (British Caucasians of at least 60 years) is somewhat higher than the mean debrisoquine MRs in two previous sets of data on Caucasian volunteers (Daly *et al.*, 1991, mean debrisoquine MR less than 1.0 for N = 73 volunteers; Sachse *et al.*, 1997, mean debrisoquine MR 3.54 for 133 debrisoquine-phenotyped German Caucasians). It is difficult to calculate whether or not the difference between the mean MR in this study does differ significantly from the mean MR in the Sachse *et al.* (1997) study owing to the raw data not being reported in the latter study. There was a trend towards an increased percentage of heterozygous individuals in the elderly Caucasians as compared to the American Caucasians studied by Daly and colleagues (Leathart *et al.*, 1998; mean age of sample not given), and the sample of Laforest *et al.* (mean age 55.0 years). However, this trend was not seen in comparison with my French young (mean age 26.15 years) Caucasian sample, and the sample of Sachse *et al.* (mean age 40 years in males, 48 years in females). This

relatively high overall mean debrisoquine MR may reflect the relatively high mean MR for subjects with one functional *CYP2D6* gene. This could either reflect the presence of an intermediate metabolising allele (e.g. *CYP2D6*2*) in my elderly cases with relatively high frequency, or reduced CYP2D6 activity in the heterozygous state due to an age-related reduction in CYP2D6 hepatic capacity. If the latter were the case, then this would be the first study to report results consistent with this, Kinirons and Crome (1997) having noted reduction in other hepatic cytochrome P450s (CYP3A4/5, CYP1A2, CYP2C9, and CYP2C18/19) with increasing age, but not having observed a reduction in CYP2D6 capacity.

It is also of note that there were 4 individuals in this study with debrisoquine MR < 0.20: three with MRs of zero (i.e. only the metabolite detected, genotypes **1x2/*1*, **1/*1*, and **1/*4*), and one with an MR of 0.15 (genotype **1/*4*). The Løvlie *et al.* cyp-17/cyp-32 assay hence detected only one out of these 4 individuals as being positive for a gene amplification event.

A study of 61 unrelated Swedish subjects with debrisoquine MR less than 0.2 revealed *CYP2D6* amplification in 14 (23%), and of those with MR less than 0.1, 40% had *CYP2D6* amplification (Dahl *et al.*, 1995a). The authors therefore concluded that there must be variation in *CYP2D6* other than gene amplification to account for high CYP2D6 activity. This was then followed up by Løvlie *et al.* (2001), who screened 13 UMs and 4 EMs for variants in exon 1 and flanking sequences of *CYP2D7P*, and the promoter region and 5' coding sequence of *CYP2D6*. (The above region of *CYP2D7P* was selected as *CYP2D7P* has an insertion of a single T at position 137 in exon 1, leading to a disrupted reading frame, which classifies it as a pseudogene – the

hypothesis was that gene-conversion events could have converted *CYP2D7* back to a *CYP2D6*-like sequence, giving an allele effectively equivalent to *CYP2D6* duplication.) All individuals tested were homozygous for the T_{137} insertion, but five variants from the 5'-end of *CYP2D6* were selected for further analysis (representing most of the variation observed, based on estimated haplotypes) in a further 27 UMs and 77 EMs. Data analysis showed that the 31A allele of the 31G>A ($Val_{11}Met$) SNP was significantly more frequent in the UMs ($P = 0.04$), and one of the haplotypes with this variant constituting 23.5% of haplotypes in the UM group as opposed to 4.2% in the EM group ($P = 0.03$). However, the authors noted that the results were only of borderline significance and not corrected for multiple testing. Indeed, a subsequent functional study showed that the functional activity and level of expression of recombinant CYP2D6.35 (positive for the 31G>A substitution) was comparable to that of wild-type enzyme (Allorge *et al.*, 2001).

Løvlie *et al.* (2001) also found a non-significant over-representation of the G-allele of a –1584C>G promoter polymorphism in the UM group, consistent with the findings of Zanger *et al.* (2001) that the –1584G variant was associated with significantly higher microsomal protein expression in liver biopsy samples. Therefore, it may not be sufficient to simply genotype for gene duplication/amplification events in order to identify UMs.

3.2 Typical antipsychotics (TAs) and CYP2D6

3.2.1 Introduction

CYP2D6 contributes significantly to the pharmacokinetics of most typical antipsychotics (Dahl and Bertilsson, 1993). Up to 30% of patients with schizophrenia who are prescribed typical antipsychotics are treatment-resistant (Kane *et al.*, 1988). The term treatment-resistant includes those who are treatment-refractory (show inadequate clinical response) and those who are treatment-intolerant (exhibit adverse responses).

Two patients with CYP2D6 UM status have been described for whom tricyclic antidepressants at doses beyond the usual therapeutic range were required in order to achieve a therapeutic response (Bertilsson *et al.*, 1993). One of these was first described by Bertilsson *et al.* (1985): a depressed patient with a very low debrisoquine MR (0.07), in whom nortriptyline doses up to 300-500 mg daily (the British National Formulary, or BNF, recommended maximum dose being 150 mg) were required to reach plasma levels of nortriptyline within the therapeutic range. Hydroxylation of nortriptyline to 10-hydroxynortriptyline (the major metabolic pathway of nortriptyline) is catalysed by CYP2D6 (Mellström *et al.*, 1981; Nordin *et al.*, 1985), and concentrations of 10-hydroxynortriptyline 3 times higher than normal were observed in this patient. The same group later showed that this patient carried a duplication of CYP2D6 (Bertilsson *et al.*, 1993), and also reported another patient with CYP2D6 gene duplication in which a clomipramine dose of 225 mg yielded a clomipramine level of 150 nM and desmethylclomipramine less than 100 nM, both much lower than expected, and required 300 mg (BNF maximum 250 mg) of clomipramine to produce an adequate

clinical response. A third patient has been described with extremely rapid metabolism of mianserin (also metabolised by CYP2D6), in which 13-fold amplification of CYP2D6 was found. There is a case report of the impact of CYP2D6 UM status on the metabolism of risperidone (Guzey *et al.*, 2000). However, when I began my study, there were no systematic case-control studies of the effect of CYP2D6 UM status on response to antidepressants or antipsychotics.

Treatment with typical antipsychotics can result in a variety of adverse effects. If these are severe, this may result in intolerance, and hence the need to change to an atypical antipsychotic, such as clozapine. The adverse effects that are most likely to cause typical antipsychotic intolerance are extra-pyramidal side-effects (EPS). These include: drug-induced parkinsonism, dystonia, dyskinesia, and akathisia, with acute and tardive variants of the last three (Barnes, 1992).

Drug-induced parkinsonism (DIP) resembles idiopathic Parkinson's disease, which is characterised by the triad of bradykinesia, rigidity, and tremor, except that asymmetrical distribution and the classical pill-rolling tremor are less common in the drug-induced form (Lishman, 1998). Tardive dyskinesia (TD) is an abnormal involuntary movement disorder caused by sustained exposure to antipsychotics, characterised by orofacial dyskinesia and choreoathetoid movements of the trunk and limbs. Risk factors for TD include: high lifetime antipsychotic exposure, especially to a high dopamine D_2-receptor potency antipsychotic, the presence of movement disorder (both subtle movement disorder prior to treatment, and acute EPS, especially drug-induced parkinsonism), negative symptoms, cognitive impairment, diabetes, alcohol or drug abuse, and, in most studies, age (Wolf *et al.*, 1983; Kane *et al.*, 1986; Woerner *et*

al., 1991; Caliguiri *et al.*, 1991; Waddington, 1995a; van Os *et al.*, 1997; Muscettola *et al.*, 1993 and 1999). Indeed, a recent study indicates that the prevalence of involuntary movements approaches 100% over a lifetime trajectory for individuals with schizophrenia chronically medicated with typical antipsychotics (Quinn *et al.*, 2001).

An association between CYP2D6 PM status and concentration-dependent adverse effects of typical antipsychotics, such as sedation, postural hypotension, and antimuscarinic side-effects, has been found (Spina *et al.* 1992). Meta-analysis of the literature has revealed that CYP2D6 PM status is associated with a small increase in risk of Parkinson's Disease (McCann *et al.*, 1997; Christensen *et al.*, 1998; Rostami-Hodjegan *et al.*, 1998). Subsequent studies have, however, yielded conflicting results, with positive associations being mainly found for the *CYP2D6*4* allele (Atkinson *et al.*, 1999; Bon *et al.*, 1999; Stefanovic *et al.*, 2000), although not invariably (Harhangi *et al.*, 2001), and not for other PM/IM alleles (Sabbagh *et al.*, 1999; Nicholl *et al.*, 1999; Joost *et al.*, 1999; Ho *et al.*, 1999; Maraganore *et al.*, 2000). It has been suggested that the apparent *CYP2D6*4* effect is an age-related artefactual association (Payami *et al.*, 2001). Some studies (Armstrong *et al.*, 1997; Andreassen *et al.*, 1997; Kapitany *et al.*, 1998; Ohmori *et al.*, 1998; Vandel *et al.*, 1999; Scordo *et al.*, 2000; Topic *et al.*, 2000), but not all (Arthur *et al.*, 1995; Hamelin *et al.*, 1999; Ohmori *et al.*, 1999), have found associations between CYP2D6 metaboliser status and antipsychotic intolerance or EPS including DIP and/or TD.

3.2.2 Aims

1 To test the hypothesis that patients with schizophrenia who were refractory to treatment with typical antipsychotics would be more likely to be ultrarapid

metabolisers, as compared with patients who responded to typical antipsychotics. (If the hypothesis were confirmed, it could form the rationale for a pre-prescribing genotyping assay to predict patients who would be less likely to respond well to typical antipsychotics at standard doses, and therefore assist the process of clinical dose finding and/or the more rapid progression of such patients on to an atypical antipsychotic not subject to the CYP2D6 polymorphism.)

2 Given some indication of an association between CYP2D6 metaboliser status and susceptibility to adverse effects of antipsychotics, especially DIP and TD, but inconsistency in the findings, my aim was to clarify the nature of the apparent associations found. In addition, my studies differ from that of most previous authors in that I genotyped not only for non-functional alleles, but also for the presence of gene duplication/amplification (*i.e.* UM status), which allowed me to determine whether or not there were associations with the number (N = 0-3) of functional *CYP2D6* genes.

3.2.3 CYP2D6 UM status and failure to respond to TAs

This study has already been published (Aitchison *et al.*, 1999b).

3.2.3.1 Methods

Two hundred and forty-six patients treated with clozapine, resistant to treatment with typical antipsychotics, were collected (R.W. Kerwin and J. Munro, South London and Maudsley NHS Trust). Of these, 235 were refractory to typical antipsychotic treatment, and were used in this study (see section 2.1.2.3 for sample details). The comparison

group, non-refractory to TAs, comprised 73 patients from the Maudsley and Bethlem Royal Hospitals NHS Trust. Of these, 66 were collected by P.Wright, and 7 were collected by Dr S. Smith (Section 2.1.2.1). Six of the seven subjects had a clinical diagnosis of schizophrenia or schizoaffective disorder, the seventh had severe depressive disorder with psychotic features. Six had been treated with the equivalent of 100 mg chlorpromazine daily, while the remaining patient received 15 mg flupenthixol decanoate fortnightly, the equivalent of approximately 75 mg chlorpromazine daily. As the patients were treated with a variety of antipsychotics, we converted all the prescriptions to chlorpromazine equivalents according to BNF guidelines, in order to assess whether or not there was a relationship between the magnitude of the dose and the CYP2D6 genotype.

Ethical Committee approval was obtained for the study on all subjects, and, as there were insufficient numbers of non-Caucasians in the sample for the analysis to be informative, all non-Caucasians were excluded.

DNA was extracted from blood collected in EDTA tubes using the Nucleon II kit, and *CYP2D6* gene amplification was detected by the long-PCR method of Løvlie *et al.*, 1996 (Section 2.2.3.2). As cases had been described in which there are extra copies of a non-functional or null *CYP2D6* allele (Løvlie *et al.*, 1996; Masimirembwa *et al.*, 1993; Sachse *et al.*, 1997), it was necessary to assay for non-functional *CYP2D6* alleles as well as for the presence of a gene amplification event in order to confirm ultrarapid metaboliser status. I therefore assayed for the *CYP2D6*3*, *CYP2D6*4*, and *CYP2D6*5* null alleles, which would be predicted to detect 90 to 95% of null alleles in a European Caucasian population (Heim *et al.*, 1990; Broly *et al.*, 1991; Dahl *et al.*, 1992). The

methodology for *CYP2D6*3-5* genotyping and the gene amplification assay by the modified version of the Løvlie *et al.* (1996) assay was as described in Section 3.1.2.1.1. Two cases were positive on both the *CYP2D6*4* and the gene amplification assays. These were further tested to determine whether the null or the wild type allele was duplicated as described by Sachse *et al.*, (1997; Section 2.2.3.2): a further gene amplification assay was performed with primers as described by Johansson *et al.* (1996), giving a 10kb amplicon in cases positive for a gene amplification, which was then subjected to a nested PCR followed by digestion with *Hph* I.

The results were analysed using SPSS for Windows and EpiInfo Version 6 (Section 2.3).

3.2.3.2 Results

CYP2D6 genotype and deduced phenotype for the 235 subjects refractory to treatment with typical antipsychotics versus the 73 responsive to typical antipsychotics are given in Table 3.5. Of the 235 treatment-refractory subjects, 4 (1.7%) cases were positive on the duplication assay. However, two cases yielded a *CYP2D6*4/wt* result with the *CYP2D6*4* assay and a positive result with the duplication assay; these cases were found to represent duplications of the *CYP2D6*4* allele. As these cases possessed only one functional copy of *CYP2D6*, they were deduced to be phenotypically equivalent to heterozygous null cases and hence EMs, not UMs. Therefore only 2 out of 235 cases (0.9%) were positive for duplication of a wild type allele. In contrast, of the sample of 73 non-refractory patients, 3 (4.1%) were positive for the duplication assay, of which none were positive for the null alleles tested. The results were therefore in the opposite direction to that predicted by our hypothesis, but they did not reach significance:

Fisher's exact test was performed, comparing the presence or absence of UM status in the 2 clinical groups, which gave a 2-tailed *P* value of 0.091. (Chi square was not appropriate here, with the number of cases being less than 5; with Fisher's exact test the 1-tailed and 2-tailed *P* values were identical, but the value is reported as 2-tailed as the results were in the opposite direction to that predicted by the hypothesis).

Table 3.6 shows the distribution of the alleles with allele frequencies in the 2 clinical groups, with the results being reported under the assumption that the duplication allele is present in the heterozygous state. If this allele were present in a homozygous state in all cases in which it was found, then the frequencies of the wild type and null alleles in the treatment-refractory group would be unchanged, while that of the duplication allele would be doubled at 0.0085; the frequencies of the wild type, null, and duplication alleles in the non-refractory group would be 0.71, 0.25, and 0.041 respectively. However, as in the paper by Johansson *et al.* (1996) none of their cases with a duplication allele were homozygous for this variant, I assumed that all of the cases are heterozygous. The frequencies of the *CYP2D6*4*, *CYP2D6*5*, and *CYP2D6*3* alleles respectively in the sample of 235 and 73 were: 0.223, 0.024, and 0.018 (in the sample of 235), and 0.219, 0.02, and 0.02 (in the sample of 73). Comparing the presence or absence of the duplication allele in the 2 groups, Fisher's exact test gave a 2-tailed *P* value of 0.10 (Table 3.6).

Table 3.5 CYP2D6 genotype and deduced phenotype in subjects refractory to treatment with typical antipsychotics and non-refractory to treatment with typical antipsychotics; numbers of cases given with percentages in parentheses

	EM (wt/wt) or (wt/mut)	PM (mut/mut)	UM (dup of wt)
Refractory to TAs (n = 235)	220 (93.6)	13 (5.5)	2 (0.9)*
Non-refractory to TAs (n = 73)	67 (91.8)	3 (4.1)	3 (4.1)*

TAs, typical antipsychotics, *wt*, wild type, *mut*, non-functional mutant or null allele (includes 2 duplications of *mut* in the EM group), dup of *wt*, duplication of wild type allele. *Two-tailed *P*-value for presence or absence of UM status in refractory versus non-refractory group = 0.09 (Fisher's exact test); odds ratio = 0.2, 95% confidence limits 0.02-1.80

Table 3.6 Distribution of *CYP2D6* alleles in the treatment-refractory and non-refractory groups; allele numbers given, with frequencies in parentheses. The *CYP2D6*4 x 2* allele is included in the null alleles

	Wild type	**Null**	Duplication of wild type
Refractory to TAs	343 (0.73)	97 (0.26)	2 (0.0043)*
Non-refractory to TAs	103 (0.72)	37 (0.26)	3 (0.021)*

TAs, typical antipsychotics.*Two-tailed *P*-value for presence or absence of duplication allele in refractory versus non-refractory groups = 0.10 (Fisher's exact test); odds ratio = 0.21, 95% confidence limits 0.02-1.88.

3.2.3.3 Discussion

I did not find an excess of ultrarapid metabolisers in subjects refractory to treatment with typical antipsychotics. On the contrary, only 2 out of 235 (0.9%) treatment-refractory cases were positive for duplication of a wild-type allele, while 3 out of 73 (4.1%) non-refractory cases were genotyped as ultrarapid metabolisers. This gives a trend ($P = 0.091$, Fisher's exact test) towards an excess of ultrarapid metabolisers in the non-refractory group of patients. However, both percentages are within the range for ultrarapid metabolisers in Caucasian populations (Agúndez *et al.*, 1995; Jerling *et al.*, 1994; Dahl *et al.*, 1995a; Sachse *et al.*, 1997). Two cases of *CYP2D6*4* duplication were found, which is the second time this has been reported in Caucasians, Sachse and colleagues (1997) having provided the first report.

The results demonstrate that ultrarapid hydroxylation by CYP2D6 of typical antipsychotics is not a major cause of failure to respond to treatment with these agents. There are at least 5 possible explanations for this surprising result. Firstly, I could have failed to find a significant result when there is in fact a significant association of ultrarapid hydroxylation either with treatment-refractory status (the direction of the original hypothesis), or with treatment non-refractory status (the direction of the trend found). The odds ratio (OR) for UM status, counting the treatment-refractory group as the "diseased state" and the non-refractory group as the "non-diseased state," was 0.2, with exact lower and upper 95% confidence limits of 0.02 and 1.8 respectively (Table 3.5). This means that ultrarapid metaboliser status is less associated with being treatment-refractory than with being non-refractory, with the range extending to being more associated with treatment-refractory. Of note the 2 patient sample groups are unequal in size; if we had had as many in the treatment non-refractory group and the

percentage of ultrarapid metabolisers in this group had remained the same as in my current findings, then I would have found 10 ultrarapid metabolisers in the non-refractory group, which would have given a chi square of 5.47, a p value of 0.019, and an OR of 0.19, with exact lower and upper limits of 0.02 and 0.92 respectively. In this scenario a significant result in the opposite direction to our original hypothesis would have been found.

Secondly, in both of my groups of subjects, the dose of antipsychotic was titrated by the prescribing consultants according to clinical effect. This could obscure any pharmacogenetic effects, *i.e.* ultrarapid metabolisers could be receiving doses above or at the upper end of the normal prescribed range, and then respond as if they were extensive metabolisers. However, for the non-refractory group one of the patients with *CYP2D6* duplication was on only 30mg flupenthixol decanoate 2 weekly (equivalent to 150mg chlorpromazine daily, *i.e.* a low dose).

Thirdly, although CYP2D6 is known to contribute to the pharmacokinetics of many typical antipsychotics (Dahl and Bertilsson, 1993), the specific contribution is different for different antipsychotics, and other cytochromes are involved. CYP2D6 is involved in the first pass metabolism and systemic elimination of perphenazine (Dahl-Puustinen *et al.*, 1989), and the systemic elimination of zuclopenthixol (Dahl *et al.*, 1991). For these drugs, high CYP2D6 activity would be expected to lead to lower serum levels of the drugs, and hence possible therapeutic resistance. Although inhibition studies demonstrated that CYP2D6 is likely to be involved in the metabolism of chlorpromazine (Inaba *et al.*, 1985; Spina *et al.*, 1991), Muralidhan and colleagues (1996) showed that CYP2D6 makes a relatively minor contribution to the large

interindividual variability seen in plasma chlorpromazine levels.

The systemic elimination of haloperidol has been shown by Llerena and colleagues (1992) to be dependent on CYP2D6 activity, and although early reports showed that CYP2D6 catalysed the oxidation of reduced haloperidol back to haloperidol (Chakraborty *et al.*, 1989; Tyndale *et al.*, 1991), other work was not consistent with this (Young *et al.*, 1993), and recent reports (Fang *et al.*, 1997; Pan *et al.*, 1998) have demonstrated that CYP3A4 is the primary enzyme involved in this step in the metabolic pathway. The steps in the metabolism of haloperidol in which CYP2D6 is involved are at present unclear, but, consistent with the results of Llerena and colleagues (1992), Nyberg *et al.* (1995) showed that a CYP2D6 poor metaboliser had higher concentrations of plasma haloperidol throughout a 4 week treatment period with haloperidol decanoate as compared with 7 CYP2D6 extensive metabolisers. Although none of the subjects in the study of Nyberg *et al* was an ultrarapid metaboliser, it would be logical to assume that a UM would have low plasma haloperidol levels. Suzuki and colleagues (1997) studied the correlation between CYP2D6 genotype and steady-state plasma concentrations (C_{ss}) of haloperidol and reduced haloperidol in a group of 50 Japanese patients with schizophrenia. They found that the mean C_{ss} of haloperidol was significantly higher ($p<0.05$) in the patients with 1 mutant allele compared to those with no mutant alleles, and that the mean C_{ss} of reduced haloperidol was significantly higher ($p<0.05$) in the patients with 1 or 2 mutant alleles compared to those with no mutant alleles. They therefore suggested that the C_{ss} of reduced haloperidol was more dependent upon CYP2D6 activity than the C_{ss} of haloperidol. However, although they did not find a significant difference between the mean C_{ss} of haloperidol in patients with 2 mutant alleles compared to those with no mutant alleles, it is of note that in this

study the patients with 2 mutant alleles were either homozygous for the *CYP2D6*10* allele (n = 4), which is associated with reduced but not absent CYP2D6 activity, or were compound heterozygotes for the *CYP2D6*10* and *CYP2D6*5* alleles (n = 2). Hence no patient actually had 2 CYP2D6 null alleles. Lane *et al.* (1997) examined the relationship between CYP2D6 phenotype (as measured by dextromethorphan/dextrorphan metabolic ratio) and haloperidol disposition in 18 newly hospitalised Chinese patients with schizophrenia. Despite the fact that no PMs were found in this study, significant correlations between the metabolic ratio and plasma haloperidol concentration, reduced haloperidol concentration, and reduced haloperidol/haloperidol ratios were found. In a preliminary report of a larger study, Schmider *et al.* (1998) investigated therapeutic drug monitoring data in 178 patients versus CYP2D6 genotype and found that PMs had significantly higher reduced haloperidol but not haloperidol concentrations compared to patients with 1 or no mutant alleles. The suggestion of Suzuki *et al.* (1997) that CYP2D6 affects reduced haloperidol levels at steady-state to a greater extent than haloperidol levels might therefore be correct. However, Suzuki *et al.* also suggested, based on the work of Tyndale and colleagues (1991), that CYP2D6 catalyses the oxidation of reduced haloperidol back to haloperidol. As already outlined above, more recent work is not consistent with this (Young *et al.*, 1993; Fang *et al.*, 1997; Pan *et al.*, 1998), although the precise step in the metabolism of reduced haloperidol in which CYP2D6 is involved is at present unclear. Young *et al.* (1993) showed that reduced haloperidol was the preferred form in the plasma after the administration of a single dose of either haloperidol or reduced haloperidol to healthy volunteers. A negative correlation between clinical response and reduced haloperidol levels or reduced haloperidol/haloperidol ratios has been observed (Bareggi *et al.*, 1990); it is possible

that ultrarapid metabolisers of CYP2D6 could have lower reduced haloperidol levels and hence a better clinical response. This would be consistent with the trend that we have found for an excess of UMs in the non-refractory group. However, Lane *et al.* (1997) did not find a correlation between response and reduced haloperidol levels, reduced haloperidol/haloperidol ratios, or haloperidol levels. This is consistent with analyses by other authors (Chang *et al.*, 1992; Altamura *et al.*, 1993).

In the case of thioridazine, CYP2D6 catalyses the formation of mesoridazine, a metabolite *with antipsychotic activity* (von Bahr *et al.*, 1991), and may be involved in the generation of another active metabolite, sulphoridazine. Extensive metabolisers have been shown to have higher peak levels of mesoridazine and sulphoridazine than poor metabolisers after a single oral dose, with lower levels of thioridazine. The total serum concentrations of substances with antipsychotic activity at steady-state will be determined by the relative magnitudes of the equilibrium constants of all the reactions in the metabolic pathway; these equilibrium constants and the relative antipsychotic potencies of the different active metabolites are unknown. It is therefore difficult to predict the effect of ultrarapid metaboliser status on clinical response to thioridazine.

Both of our groups of patients had been treated with various typical antipsychotics; it is therefore possible that I failed to show a correlation in one direction or the other as effects with some antipsychotics versus other antipsychotics cancelled each other out. Furthermore, it is possible that some patients in the treatment-refractory group were treated with agents whose levels are not significantly affected by CYP2D6 genotype (such as chlorpromazine). However, I would emphasise that a significant result would be unlikely to be obscured by either of the above possibilities as the numbers of

individuals with duplications is *very low in both* the treatment-refractory and non-refractory groups. It is also of note that the subject on zuclopenthixol who is a UM is clinically stable on a low dose.

Fourthly, several of the above studies (especially those on normal volunteers) were single-dose pharmacokinetic analyses; single dose effects may differ markedly from those seen at steady-state in a situation of pharmacological adaptation (Grahame-Smith, 1997). However, the work of Nyberg *et al.* (1995), Suzuki *et al.* (1997), Lane *et al.* (1997), and Schmider *et al.* (1998) was conducted on patients at steady-state. Furthermore, Jerling *et al.* (1996) conducted a study on patients during continuous treatment and CYP2D6 genotype was shown to significantly predict the oral clearance of perphenazine and zuclopenthixol (patients with 2 CYP2D6 null alleles having a significantly lower clearance than those with one or no mutant alleles).

Finally, other factors may contribute towards non-response to medication, including non-compliance, pharmacodynamic factors, other biological factors, and psychosocial factors. Non-compliance occurs in up to 50% of patients on neuroleptics (Bebbington *et al.*, 1995). Pharmacodynamic factors have been implicated in the clinical response to clozapine, an atypical antipsychotic (Arranz *et al.*, 1995); it may be that other pharmacodynamic factors (*e.g.* D_2 receptor variants) are involved in the response to typical antipsychotics. Lieberman and colleagues (1996) and Van Os *et al.* (1996) have reviewed predictors of outcome in psychotic illness and concluded that factors such as longer duration of untreated illness, and structural brain abnormality on CT or MRI predict unfavourable outcome, while living in a low "expressed emotion" environment is one of the predictors of a favourable outcome.

I did not find an association between ultrarapid metaboliser status and being treatment-refractory to typical antipsychotics. On the contrary, I found a trend towards an association between UM status and being non-refractory to TAs, which could have reached significance had the non-refractory group been equal in size to the refractory group.

Nonetheless, I have not excluded the possibility that UM status could lead to failure to respond to a standard dose of some TAs and other drugs metabolised at least partially by CYP2D6, *e.g.* risperidone. In the case of risperidone, there has been a report describing two individuals treated with risperidone (4 mg/day) who had a lack of therapeutic response and lower than expected concentrations of risperidone for that dose, and who were found to be ultrarapid metabolisers on genotyping (Güzey *et al.*, 2000). However, Güzey and colleagues note that the concentration of 9-hydroxyrisperidone (which also has antipsychotic activity) was as expected or only slightly lower than expected, so that the concentration of the active moiety (i.e. risperidone plus 9-hydroxyrisperidone) would only be slightly lower, but not considerably lower than expected (see also Scordo *et al.*, 1999). The authors also note that they were not able to identify the exact number of copies of the active allele (using the assay developed by Løvlie *et al.*, 1996).

I did not genotype for the –1584C>G promoter SNP (Section 3.1.1.1), which may also be associated with UM status. However, as only 0.5% to 7% of Caucasians are UMs, unless UM status were associated with the psychotic illnesses for which the antipsychotics were prescribed (in this study, mainly schizophrenia), then as treatment

resistance occurs in up to 30% of cases of schizophrenia, then this factor would be unlikely to account for the majority of cases of treatment resistance. In summary, my data are not consistent with UM status being a major cause of failure to respond to typical antipsychotics, but are consistent with UM status being weakly associated with response to TAs, especially in the case of haloperidol.

3.2.4 Number of functional CYP2D6 genes and adverse effects of TAs

3.2.4.1 Sib pair and twin pair

3.2.4.1.1 Methods

The subjects were as described in section 2.1.2.2. The sib pair and twin pair had suffered intolerance to a variety of antipsychotics, the details of their adverse responses being summarised in Table 3.7.

The twins were monozygotic. There were no other siblings, and no other family member with any psychiatric or medical condition. Neither twin had ever used alcohol or illicit drugs.

Twin 1 (DNA number 2134) first saw a psychiatrist in October 1988, aged 25, after she had become increasingly paranoid and withdrawn. Later she described passivity phenomena and third person auditory hallucinations. She was diagnosed as suffering from paranoid schizophrenia, and treated with trifluoperazine 10mg. After 10 days this treatment was discontinued due to "spasms" and profuse sweating. Perphenazine was then tried but this produced a rash and akathisia, and also could not be tolerated. During

Table 3.7 Summary of adverse effects of antipsychotics experienced by twin pair and sib pair

Subject	Antipsychotic(s)	Date	Adverse effect
Twin 1 (2134)	Trifluoperazine 10 mg	1988	"Spasms" and profuse sweating
	Perphenazine	1988	Rash and akathisia
	Sulpiride 200 mg + chlorpromazine 75 mg	1989	Orthostatic hypotension
	Pimozide 4 mg	1990	Parkinsonism and dystonia
	Fluphenazine decanoate 12.5 mg	1991	Dystonia (right limb)
	Haloperidol 20 mg	1991	Oculogyric crisis
	Clozapine 150 mg	1991-94	Orthostatic hypotension, neutropenia
Twin 2 (2135)	Chlorpromazine 75 mg + sulpiride 400 mg	1989	Orthostatic hypotension, akathisia, dystonia
	Trifluoperazine 80 mg	1990	Dystonia and dyskinesia
	Haloperidol 9 mg	1990	Parkinsonism and orofacial dyskinesia
	Clozapine 300 mg	1991-'94	Neutropenia
	Risperidone	1994	Diarrhoea and vomiting
	Loxapine	1994	Headaches
	Fluphenazine 3 mg	1994	Nil, but ineffective
	Pimozide 4 mg	1994	Dystonia (lower limbs)
	Thioridazine 40 mg	1994	Parkinsonism
Sib 1 (2007)	Trifluoperazine 10 mg	1986	Dystonia (upper limb)
	Chlorpromazine 400 mg + sulpiride 400 mg	1987	Orthostatic hypotension
	Pimozide 4 mg	1988	Akathisia
	Trifluoperazine 12 mg spansules	1988	Dystonia and akathisia
	Risperidone	1993	Neutropenia and dystonia
	Thioridazine 300 mg + sulpiride 400 mg	1994	Postural hypotension and impotence
Sib 2 (2206)	Chlorpromazine	1993	Nil
	Sulpiride 800 mg	1994	Orofacial dyskinesia, neutropenia
	Risperidone 4 mg	1994	Oversedation, neutropenia, dystonia
	Trifluoperazine	1995	Nil

the next year she was treated with sulpiride 200mg and chlorpromazine 75mg daily, which was associated with orthostatic hypotension. In July 1990 trifluoperazine 10mg, with procyclidine, was reintroduced as she had become catatonic. She was unable to tend for herself, and virtually immobile. There was little improvement so pimozide 4

mg was added in December 1990. This resulted in dystonia and parkinsonism. In view of the multiplicity of problems twin 1 was admitted to hospital and tried on fluphenazine decanoate 12.5 mg. However this led to an acute limb dystonia. Haloperidol 20 mg resulted in an oculogyric crisis, so in July 1991 clozapine was started. This also resulted in orthostatic hypotension, but treatment with clozapine 150 mg was continued as she was felt to have made a better clinical response than she had made to any other antipsychotic up until that time. Unfortunately, after a succession of "amber" results (*i.e.* relatively low neutrophil count), in May 1994 she developed a neutropenia and by March 1995 had again developed a neutropenia, with the result that clozapine had to be withdrawn. Within three weeks she was in catatonic stupor which only resolved when clozapine was cautiously reintroduced. Later she was entered into a double-blind trial of sertindole. Unfortunately she lapsed once more into a catatonic state prior to the commencement of active treatment. Finally, a combination of thioridazine and low-dose loxapine brought some normalisation of her mental state, enabling her to become self-caring even though she remained deluded and hallucinated.

Twin 2 DNA number (2135) first became ill a year after her sister, in 1989, aged 26. She had persecutory delusions and auditory and visual hallucinations. She also would collapse to the floor, shaking. She was treated with chlorpromazine 75 mg and sulpiride 400 mg, and like her sister, had orthostatic hypotension on this combination, and also developed dystonia and akathisia. In 1990 trifluoperazine was tried, up to a maximum dose of 80 mg. This led to dystonic and dyskinetic movements, and was then substituted with haloperidol 9 mg, which led to orofacial dyskinesia and parkinsonism. In July 1991 twin 2, like her co-twin, was commenced on clozapine, titrating up to a dose of 300mg daily. Again, the clozapine seemed to help greatly but a worsening

neutropenia led to a "red alert" being given by the Clozaril Patient Monitoring Service in December 1993, and hence withdrawal of clozapine. In early 1994 twin 2 was prescribed risperidone and loxapine. The former led to diarrhoea and vomiting with subsequent weight loss, and the latter produced headaches. In March 1994 fluphenazine 3 mg was tried, but deemed to be "not effective" by PT and her family after only a month's trial. Following this, pimozide 4 mg was prescribed, but resulted in lower limb dystonias. By July 1994 thioridazine 40 mg was the only neuroleptic employed. The family maintained thioridazine "didn't work" and twin 2 complained of a stiff face. The responsible psychiatrist however felt clinically there was a partial response to thioridazine. In July 1995 she entered a double-blind trial of sertindole. As part of the trial, her psychopathology and any movement disorder were assessed using the PANSS (positive and negative symptom scale); Simpson-Angus scale (for extrapyramidal side effects); and AIMS (abnormal involuntary movements scale). Her pre-treatment and post-treatment Simpson-Angus and AIMS scores were 6 and 0, and 9 and 0 respectively. Her mental state also improved on the sertindole (pre-treatment PANSS score 50, post-treatment PANSS score 21), and she was discharged home on this medication.

Sib 1 DNA number (2007) was 29 years, sib 2 DNA number (2206) 23 years at the time of sampling, and they had a sister at 27 years. Both sibs were diagnosed with schizophrenia. There was no other family history of mental disorder.

Sib 1 was aged 20 when first seen by a psychiatrist, in 1986. A change in his behaviour had been noted for the previous 5 months, and at assessment he was deluded and felt to have symptoms of schizophrenia. He was also taking a variety of recreational drugs at

this time. Trifluoperazine 10 mg was prescribed, which produced an upper limb dystonia. After a period unmedicated he relapsed in July 1987, and was commenced on chlorpromazine to 400 mg daily with sulpiride 400 mg. Despite some orthostatic hypotension, he showed some clinical improvement. Unfortunately, by December 1987 he had again stopped his neuroleptic treatment. Pimozide 4 mg was then commenced, resulting in akathisia. After non-compliance again, trifluoperazine (12 mg spansules) was again prescribed, which resulted in an upper limb dystonia and akathisia. Nevertheless he remained on this treatment until April 1993, with only partial resolution of his psychotic symptoms, but another episode of self-harm at that time led to an involuntary admission. It was then that he was put on risperidone 4 mg, which resulted in limb dystonia and neutropenia (1.4 x 10^9/l). The haematologist consulted performed a bone marrow biopsy and felt this low count was because of neutrophil margination, rather than agranulocytosis. Eventually it was decided to change his medication to thioridazine 300 mg and sulpiride 400 mg daily. The thioridazine resulted in orthostatic hypotension and complaints of impotence, and so the dose was halved. Sib 1 has remained partially treated on this combination since.

Sib 2 (2206) first saw a psychiatrist in August 1993, aged 21, and it was noted that he had been behaving oddly for the previous 5 weeks. He also gave a history of excessive drug use, including ecstasy, cannabis, and LSD. At assessment he was deluded and admitted to hospital involuntarily. He settled quickly on chlorpromazine, without any documented adverse events. However, he defaulted from follow-up or treatment after discharge, and when readmitted was started on sulpiride up to 800 mg daily. This had a good therapeutic effect, but his mother noticed him to be suffering from "writhing movements of the jaw" (*i.e.* orofacial dyskinesia), which sib 2 was unaware of. Routine

blood testing revealed a progressive neutropenia, down to a level of 1.3 x 10^9/l. Bone marrow biopsy was not performed in this case, and eventually it was decided to change his medication to risperidone 4 mg, which was found to be oversedative, and resulted in a limb dystonia and a neutropenia of 1.4 x 10^9/l. Trifluoperazine was then commenced, which apparently was well tolerated, although after discharge he failed to comply, and remained untreated in the community.

Debrisoquine phenotyping of the twins, the sib pair and their sister and parents was conducted as in section 2.2.1.1. Blood was collected for DNA analysis, and genotyping was performed for *CYP2D6* alleles **3-5* and gene amplification, as described in sections 2.2.3.1, and 3.1.2.1. Genotyping was performed blind to debrisoquine phenotype status.

3.2.4.1.2 Results

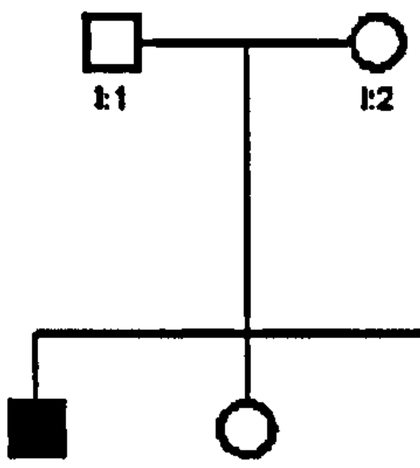
When I analysed the results of the *CYP2D6*3-5* and gene amplification genotyping, there was a genotype-phenotype discrepancy for 2134 and 2007. Under my supervision, Kopal Tandon had performed genotyping of the twins plus the sibs and their relatives for the *CYP2D6*2* (as described by Løvlie *et al.*, 2001) and the **6* allele (as described by Sachse *et al.*, 1997).

The results of the debrisoquine phenotyping and CYP2D6 genotyping including the **2* and **6* results and the medication being taken at the time of phenotyping are given in Table 3.8 and Figure 3.7.

Table 3.8 Results of debrisoquine phenotyping and CYP2D6 genotyping in the twin and sib pair samples

DNA number	MR_{deb}	Phen	*2	*3	*4	*5	*6	*dup*	*Medication at phenotyping*
2134 (twin 1)	NMD[a]	PM	**2/wt*	*wt/wt*	*wt/wt*	*wt/wt*	*wt/wt*	*wt/wt*	Thioridazine 200 mg tds, Loxapine 75 mg, Erythromycin 250 mg qds (stopped 2 days prior), Procyclidine 5 mg, Temazepam 10 – 20 mg nocte prn
2135 (twin 2)	1.55	IM	**2/wt*	*wt/wt*	*wt/wt*	*wt/wt*	*wt/wt*	*wt/wt*	Sertindole 24 mg, Potassium iodate 170 mg single dose (for SPECT scan)
2007 (sib 1)	NMD[a]	PM	**2/wt*	*wt/wt*	**4/wt*	*wt/wt*	*wt/wt*	*wt/wt*	Thioridazine 300 mg
2206 (sib 2)	6.84	IM	**2/wt*	*wt/wt*	**4/wt*	*wt/wt*	*wt/wt*	*wt/wt*	Risperidone 2mg bd
2209 (father)	0.95	EM	**2/*2*	*wt/wt*	*wt/wt*	*wt/wt*	*wt/wt*	*wt/wt*	Nil
2208 (sister)	0.62	EM	**2/wt*	*wt/wt*	**4/wt*	*wt/wt*	*wt/wt*	*wt/wt*	Nil
2207 (mother)	0.73	EM	**2/wt*	*wt/wt*	**4/wt*	*wt/wt*	*wt/wt*	*wt/wt*	Nil

MR_{deb} = metabolic ratio of debrisoquine; NMD = no metabolite detected, Phen = phenotype on basis of debrisoquine MR (PM defined by MR >12.6 or NMD, IM by MR >1.50 and less than or equal to 12.6, EM by MR less than or equal to 1.50); *dup* = *CYP2D6* gene duplication or amplification genotype; *wt* = wild type.



2209
0.95, EM
*2/*2

2209
0.73, EM
*2/*4

2007
NMD, PM
*2/*4

2208
0.62, EM
*2/*4

2206
6.84, IM
*2/*4

Figure 3.7 Genogram of family with 2 siblings affected with schizophrenia; results of debrisoquine phenotyping and *CYP2D6* genotyping and given. NMD = no metabolite detected, PM = poor metaboliser, IM = intermediate metaboliser, EM = extensive metaboliser.

3.2.4.1.3 Discussion

Sib 1 and sib 2 are both genotyped as **2/*4*, but sib 1 is phenotypically a PM, sib 2 an IM. At the time of phenotyping, sib 1 was being prescribed 300 mg thioridazine daily. Llerena *et al.* (2001) demonstrated that thioridazine is a potent CYP2D6 inhibitor, confirming the earlier results of Spina *et al.* (1991), reporting that the effect of thioridazine dosage on debrisoquine hydroxylation phenotype was dependent on *CYP2D6* genotype. All patients with homozygous wild type genotype treated with a dose of at least 150 mg/day were phenotypically PMs, while for patients with *wt/*4* genotype, a thioridazine dose of 50 mg/day was sufficient to convert phenotype to PM

(*CYP2D6*2* was not assayed for in this study). The dose of 300 mg thioridazine should therefore be more than sufficient to convert the phenotype of sib 1 (genotype **2/*4*) to PM, and is likely to account for the difference in phenotype between sibs 1 and 2.

Sib 1 may also have another mutation, on the same allele as the **2*, inherited from his father (Figure 3.7, both siblings have inherited **4* from their mother), and which is associated with reduced or absent CYP2D6 enzyme activity. Sib 2, by contrast, would be postulated to have inherited the other **2* allele from the father, consistent with a **2* allele with reduced enzyme activity as previously described. In the report by Sachse *et al.* (1998) on intermediate metabolisers, the mean MR for individuals of **2/*4* genotype was 4.40 (range 1.11 – 11.8). The debrisoquine MR for sib 2 is therefore entirely consistent with **2/*4* genotype, especially as sib 2 was prescribed risperidone, which is also known to be metabolised by CYP2D6 (compare with the sister, who was also genotyped as **2/*4*, but not on any prescribed medication at the time of phenotyping).

In the case of the twins, twin 2 was taking sertindole (which is metabolised by CYP2D6) at the time of phenotyping, and was genotyped as **2/wt*. This is consistent with the debrisoquine MR of 1.55. Twin 1 was taking thioridazine (600 mg daily) at the time of phenotyping, and, again, thioridazine prescription is likely to account for the PM phenotype, especially as the total daily dose of thioridazine was high, and the twins are monozygotic.

Of note, phenotypically, both twins and the two siblings had low CYP2D6 activity (IM or PM). Genotypically, the sibs were **2/*4* (i.e. had only one functional *CYP2D6*

allele, *i.e.* the *CYP2D6*2*, which, as previously discussed, is usually associated with reduced activity), while the twins were **2/wt*. The high incidence of adverse effects of typical antipsychotics may be due to the likely relatively low CYP2D6 activity conferred by these genotypes, rendering these individuals more susceptible to inhibition of CYP2D6 by psychotropic medications including antipsychotics. Zanger *et al.* (2001) reported that recombinantly expressed CYP2D6.2 was associated with one-third of the P450 content of recombinantly expressed CYP2D6.1. Spina *et al.* (1991) reported that there was an increased frequency (46.2%) of debrisoquine PM status in 91 patients with schizophrenia on neuroleptic monotherapy as compared with the frequency (7.5%) in 67 unmedicated healthy controls, due to the inhibitory effect of the neuroleptics on debrisoquine metabolism. In addition, thioridazine was noted as a particularly potent CYP2D6 inhibitor; 50 mg thioridazine given to 8 healthy controls converted 4 of these (two IMs, two EMs) to PM phenotype.

In this study, both sib 1 and twin 1 were being prescribed relatively high doses of thioridazine (300 mg and 600 mg daily respectively) at the time of phenotyping, and although they were genotypically the same as co-sib or co-twin, both of whom were phenotypically IMs, they were phenotypically PMs. The co-sib and co-twin at time of phenotyping were being prescribed risperidone 2 mg twice daily and sertindole 24 mg daily respectively. Risperidone is metabolised by CYP2D6 to 9-hydroxyrisperidone, and would therefore be expected to be a CYP2D6 inhibitor; sertindole is also metabolised at least partially by CYP2D6. A more definitive phenotype would have been obtained had it been possible to discontinue all medication in both members of the sib and twin pairs prior to phenotyping, but this was not clinically feasible. The genotype would be more definitive if genotyping for the -1584C>G promoter variant

were conducted, and this work is in progress. Although it is possible that sib 1 and the twins are positive for mutations not yet identified, the inhibition by thioridazine is a sufficient explanation for the difference in phenotypes between each member of the two pairs. There is a history of penicillin sensitivity in both the twins; it is also possible that they also have another enzyme deficiency, related to the penicillin allergy, and that it is the relatively low CYP2D6 activity in combination with another possible enzyme deficiency that renders them particularly sensitive to the adverse effects of antipsychotics.

Interestingly, Gill *et al.* (1997) described a case of a patient with schizophrenia who had suffered adverse effects with many different antipsychotics, awas genotyped as *CYP2D6*4/*4*, and subsequently treated successfully with a very low dose of a typical antipsychotic (chlorpromazine 50 mg daily). If the sibs and twins can be genotyped more definitively, then this may provide data consistent with the report of Gill and colleagues.

3.2.4.2 Case-control study

3.2.4.2.1 Methods

Two samples of patients were employed, for 3 case-control studies: a sample of 246 paients treated with clozapine, for whom information was available regarding history of intolerance to TAs, and a sample of 72 patients scored for drug-induced parkinsonism (DIP) and tardive dyskinesia (TD). These samples are the same as those described in Sections 2.1.2.2, 2.1.2.1, and 3.2.3.1, except that one of the 7 subjects collected by Dr S. Smith was excluded from this study as there was insufficient clinical detail in order

to enable controlling for confounding variables in the DIP and TD studies. The design of the study was to compare *CYP2D6* gene dosage in individuals with and without TA intolerance, with and without DIP, and with and without TD.

Ethical Committee approval was obtained as above, and CYP2D6 genotyping was conducted as described in section 3.2.3.1. Data were analysed by Fisher's exact test or chi-squared test (as appropriate), comparing genotype and allele data in intolerant versus non-intolerant groups, those with and without DIP, and those with and without TD. In addition, logistic regression (dependent variable: presence of typical antipsychotic intolerance, or DIP, or TD in respective studies) versus *CYP2D6* gene dosage, controlling for potential confounding variables as appropriate (see below), and multiple linear regression analysis (dependent variable: total AIMS score) was conducted. Power analyses were performed using Solo Power Analysis and NCSS 2000.

3.2.4.2.2 Results

Out of the 246 patients taking clozapine, there were a total of 26 who had a history of intolerance to typical antipsychotics (10.6%). Seventy-nine were female (32.1%), one hundred and sixty-seven male (67.9%). The mean age was 39.0 years (range 19-81 years, SD 10.57).

The *CYP2D6* genotype, deduced phenotype and history of reason for resistance to typical antipsychotics in the 246 clozapine-treated patients is given in Table 3.9. Thirteen patients (5.3 %) were homozygous for non-functional alleles (*mut/mut*) and

Table 3.9 CYP2D6 genotype, deduced phenotype and history of reason for resistance to typical antipsychotics; numbers of cases are given with percentages in parentheses

	UM (*dup* **of** *wt*)	**EM** (*wt/wt*)	**EM** (*wt/mut*)	**PM** (*mut/mut*)
Gene dosage	**3**	**2**	**1**	**0**
Refractory to TAs ($n = 220$)	2 (0.9)	113 (51.3)	93 (42.3)	12 (5.4)[a]
Intolerant to TAs ($n = 11$)	0 (0)	5 (45.5)	6 (54.5)	0 (0)[a]
Intolerant and refractory to TAs ($n = 15$)	0 (0)	10 (66.7)	4 (26.7)	1 (6.7)[a]
Frequency of intolerance	0	0.12	0.10	0.08

TAs, typical antipsychotics; *wt*, wild-type; *mut*, non-functional mutant allele, *i.e.* *CYP2D6*3*, *CYP2D6*4*, or *CYP2D6*5* (includes 2 duplications of *mut* in the *wt/mut* group); *dup* of *wt*, duplication of wild-type allele. Gene dosage refers to the number of functional *CYP2D6* genes.

[a]Two-tailed *P*-value for presence or absence of PM status in intolerant versus non-intolerant groups = 1.0 (Fisher's exact test); odds ratio = 0.69, 95% confidence limits 0.02-5.08. Chi-squared = 0.3, $P = 0.59$ for presence or absence of non-functional mutant allele in intolerant versus non-intolerant groups; odds ratio = 0.83, 95% confidence limits 0.38-1.68.

therefore classified as PMs; of these, 9 were homozygous for the *CYP2D6*4* allele, 3 were *CYP2D6*4/CYP2D6*5*, and 2 were *CYP2D6*4/CYP2D6*3*. The frequencies of the *CYP2D6*4*, *CYP2D6*5*, and *CYP2D6*3* alleles were 0.223, 0.024, and 0.018. Four cases were positive on the *CYP2D6* gene duplication assay, but of these, 2 were found to represent duplications of *CYP2D6*4*. As these cases possessed only one functional copy of CYP2D6, they were deduced to be phenotypically equivalent to heterozygous *mut* cases and hence included in this group. Two cases (0.8%) were positive for duplication of a wild-type allele and hence classed as UMs. Cases heterozygous or homozygous wild-type were classed as EMs. The genotype frequencies were in Hardy-Weinberg equilibrium.

As the PM phenotype results from homozygosity of CYP2D6 non-functional alleles (Daly *et al.*, 1991), it is logical to analyse the genotyping data using a recessive model. I also grouped together those with a history of intolerance with those with a history of being both refractory and intolerant. As can be seen in Table 3.9, in progressing from the *mut/mut* (no functional *CYP2D6* genes) to the *wt/wt* (2 functional *CYP2D6* alleles) group, there appears to be a correlation between the frequency of intolerance to typical antipsychotics and the number of functional *CYP2D6* genes. Logistic regression analysis, to determine whether or not there was a relationship between the number of functional *CYP2D6* genes ("gene dosage") and the presence of typical antipsychotic intolerance, was therefore performed. The variable "gene dosage" was given a value of 0 for the *mut/mut* genotype, 1 for the *wt/mut* genotype, 2 for the *wt/wt* genotype, and 3 for cases positive for the duplication of a wild-type allele. As in the paper by Johansson *et al.* (1996), none of their cases with a duplication allele was homozygous for this variant, I assumed that the cases positive on the duplication assay were heterozygous

for the duplication allele. However, this analysis revealed no association between gene dosage and intolerance (Wald score = 0.23, $P = 0.63$). Adding in age and gender as covariates did not change the results of the analysis significantly (Wald score = 0.25, $P = 0.62$).

The CYP2D6 genotype and deduced phenotype with respect to the presence or absence of DIP is given in Table 3.10. Thirty-five out of 66 patients (53%) met the criteria for DIP. Thirty-six out of the 66 were male (54.5%), thirty were female (45.5%). The mean age was 41.5 years (range 19-87 years, SD 17.61), the mean age of those with DIP (41.5 years) not differing significantly from the mean age of those without DIP (41.6 years). Logistic regression analysis showed no association between DIP and gene dosage (Wald score 0.55, $P = 0.46$). In addition, entering age, gender, and duration of treatment into the logistic regression as covariates revealed no significant association between these variables, and no significant change to the P value for gene dosage (age: Wald score 0.20, $P = 0.66$; gender: Wald score 0.40, $P = 0.52$; duration of treatment: Wald score 1.84, $P = 0.18$; gene dosage: Wald score 0.66, $P = 0.42$).

The CYP2D6 genotype and deduced phenotype with respect to presence or absence of TD is given in Table 3.11. Thirteen out of 72 patients (18.1%) met the criteria for RDC probable TD. Thirty-nine were male (54.2%), thirty-three female (45.8%). The mean age was 42.0 years (range 19-87 years, SD 17.3), and the mean duration of typical antipsychotic treatment was 142.9 months (range 14-396, SD 90.7). The frequencies of the *CYP2D6*4*, *CYP2D6*5*, and *CYP2D6*3* alleles were 0.215, 0.014, and 0.021 respectively. Three out of 72 (4.2%) of cases were ultrarapid metabolisers, of which none was positive for the null alleles tested. The genotype frequencies were in Hardy-

Table 3.10 CYP2D6 genotype and deduced phenotype in 66 patients with DSM-IIIR schizophrenia, with and without drug-induced parkinsonism (DIP); numbers are given, with percentages in parentheses

	UM (*dup* of *wt*)	EM (*wt/wt*)	EM (*wt/mut*)	PM (*mut/mut*)
Gene dosage	**3**	**2**	**1**	**0**
DIP present (n = 35)	0 (0)	21 (60)	14 (40)	0 (0)[b]
DIP absent (n = 31)	2 (6)	14 (45)	12 (39)	3 (10)[b]
Frequency of DIP	0	0.60	0.53	0

[b]Two-tailed *P*-value for presence or absence of PM status in those with and without DIP = 0.1 (Fisher's exact test); odds ratio 0.00, 95% confidence limits 0.00-2.10. Chi-squared = 1.46, $P = 0.23$ for presence or absence of non-functional mutant alleles in those with and without DIP; odds ratio = 0.61, 95% confidence limits 0.25-1.46.

Table 3.11 CYP2D6 genotype and deduced phenotype in patients with and without RDC probable tardive dyskinesia (TD); numbers of cases are given with percentages in parentheses

	UM (*dup* of *wt*)	**EM (*wt/wt*)**	**EM (*wt/mut*)**	**PM (*mut/mut*)**
Gene dosage	**3**	**2**	**1**	**0**
TD present ($n = 13$)	1 (8)	9 (69)	3 (23)	0 (0)[c]
TD absent ($n = 59$)	2 (3)	27 (46)	27 (46)	3 (5)[c]
Frequency of TD	0.33	0.25	0.10	0

[c]Two-tailed *P*-value for presence or absence of PM status in patients with and without RDC probable TD = 1.0 (Fisher's exact test); odds ratio = 0.00, 95% confidence limits = 0.00-11.37. Chi-squared = 3.07, $P = 0.08$ for presence or absence of non-functional alleles in patients with and without RDC probable TD; odds ratio = 0.34, 95% confidence limits = 0.06-1.23.

Weinberg equilibrium, and there was no significant difference between patients with and without RDC probable TD in the genotypic distribution. However, the allelic distribution revealed a trend towards a negative association between non-functional alleles and RDC probable TD ($P = 0.08$).

The results of the logistic regression (presence versus absence of probable TD, N = 72) and multiple regression analysis (versus total AIMS score) are given in Table 3.12. This revealed significant associations between the occurrence of RDC probable TD or total AIMS score and duration of antipsychotic treatment and DIP. There was also a significant association between gene dosage and RDC probable TD ($P = 0.04$, N = 72), with the direction of effect being an increase in the number of functional *CYP2D6* genes being associated with an *increased* risk of TD (odds ratio = 4.86). Similarly, there was a trend towards an association between gene dosage and total AIMS score of at least 6 ($P = 0.06$, N = 72, OR = 4.54). For 6 out of 72 subjects, there was information regarding the dose of antipsychotic; the mean dose, expressed in chlorpromazine equivalents, in those with TD (N = 2) was 375 mg, SD 318.20, and the mean dose in those without TD (N = 4) was 168.75 mg, SD 102.82. For these 6 subjects there was also data available regarding anticholinergic medication, which was prescribed in 1 out of 4 cases without TD, mean dose 1.35 mg (procyclidine). Restricting the analysis to those subjects that had had a second assessment using the AIMS in order to confirm TD (N = 66, of whom 11, i.e.16.7%, met the Research Diagnostic Criteria for persistent TD), there was a trend towards an association between gene dosage and RDC persistent TD ($P = 0.14$, odds ratio = 3.49). However, there was no association between gene dosage and total AIMS score for either the whole sample ($P = 0.45$), or the subset of sixty-six ($P = 0.72$).

Table 3.12 Outcome variables related to the vulnerability to TD or total AIMS score by regression analysis

	Outcome variables			
	With versus without RDC probable TD (logistic regression model)		Total AIMS score (multiple regression model)	
	Regression coefficient	*P*	Regression coefficient	*P*
CYP2D6 gene dosage	1.58	0.04	0.51	0.45
Age (years)	-0.11	0.11	-0.03	0.37
Gender	0.71	0.41	1.27	0.18
Duration of treatment with typical antipsychotics (months)	0.02	0.01	0.01	0.02
Drug-induced Parkinsonism	2.44	0.02	2.36	0.01

CYP2D6 gene dosage: number of functional *CYP2D6* genes, 0 = homozygous for a non-functional *CYP2D6* allele, 1 = heterozygous for non-functional *CYP2D6* allele, 2 = wild-type, 3 = positive for *CYP2D6* gene duplication allele.

3.2.4.2.3 Discussion

In the sample of 246 clozapine-treated patients, there was no association between CYP2D6 PM status or number of functional *CYP2D6* genes and a history of intolerance to typical antipsychotics. The sample has a power of 1.0 (allelic or genotypic comparison) at the $P < 0.05$ level to detect a medium effect size ($w = 0.30$) for the χ^2 test between the intolerant and non-intolerant groups (Cohen, 1977). With a smaller effect size ($w = 0.2$), the power remains high at 0.99 (allelic) or 0.88 (genotypic). My results are consistent with those of Hamelin *et al.* (1999), who genotyped 39 patients with schizophrenia for 5 non-functional *CYP2D6* alleles (*CYP2D6*3*, *CYP2D6*4*, *CYP2D6*5*, *CYP2D6*6*, and *CYP2D6*7*), and found that neither the one PM subject nor the EM heterozygotes differed statistically from EM homozygotes with respect to the number or severity of adverse drug reactions after at least 21 days of treatment with antipsychotics metabolised at least partially by CYP2D6 (mainly haloperidol and fluphenazine).

These results are, however, contrary to those of Spina *et al.* (1992), Chou *et al.* (2000), and Topic *et al.* (2000). Spina *et al.* (1992) estimated CYP2D6 activity by phenotyping (with the debrisoquine hydroxylation test), rather than by genotyping, and the phenotyping procedure was conducted after a minimum of one week after discontinuation of antipsychotic medication. This may not have been long enough; many typical antipsychotics are CYP2D6 inhibitors, and hence affect the phenotyping test (Spina *et al.*, 1991, Llerena *et al.*, 1993), and a minimum of 2 weeks for a washout period prior to phenotyping may be necessary, depending on the half-life of the drug involved. Hence at least some of the subjects, particularly those on higher doses of antipsychotics, who were apparently CYP2D6 PMs in the study of Spina *et al.* (1992)

may in fact have been CYP2D6 EMs. The apparent correlation between concentration-dependent adverse effects of neuroleptics and CYP2D6 metaboliser status may then have in fact reflected a correlation between the adverse effects and dose of antipsychotic. Chou *et al.* (2000) studied 100 patients on a variety of psychotropic agents at least partially metabolised by CYP2D6, rated them for adverse drug events including EPS (Simpson Angus Scale), and found a trend towards an inverse association between adverse drug events and *CYP2D6* gene dosage. However, their sample was mixed (in terms of diagnosis and drug), and the association between adverse drug events and CYP2D6 gene dosage may be stronger in the case of antidepressants than in the case of antipsychotics (Chen *et al.*, 1996; Aitchison *et al.*, 2001a; Section 3.3). Although Topic *et al.* (2000) found a positive association between homozygosity for *CYP2D6* non-functional alleles and side effects of antipsychotics, they also found an overrepresentation of *CYP2D6*4* in the subjects with schizophrenia, which is not consistent with previous findings (Spina *et al.*1992; Dawson *et al.*, 1994; Aitchison *et al.*, 1999b; Hamelin *et al.*, 1999).

Similarly, I did not find an association between CYP2D6 PM status or number of functional *CYP2D6* genes and DIP. Although Arthur *et al.* (1995) showed a correlation between the debrisoquine metabolic ratio (MR) and the severity of EPS, only one of 6 patients who was phenotypically a PM was also genotypically a PM. The authors themselves comment that the apparent relationship between MR and EPS needs to be interpreted with caution due to the effect on the phenotype of the prescribed antipsychotics. Armstrong *et al.* (1997) found that 4 out of the 5 CYP2D6 PMs (out of a total study population of 76) suffered from a movement disorder, as compared with 42% of homozygous wild-type individuals and 46% of heterozygotes; however,

because the number of PMs was small, this difference did not reach statistical significance. Moreover, they grouped together patients with DIP and TD. Although Andreasson *et al.* (1997) found a non-significant tendency for PMs to have higher DIP ratings as compared to EMs, there was no significant difference in the frequency of the PM genotype among patients with or without DIP. Vandel *et al.* (1999) studied 65 inpatients on a variety of psychotropics (tricyclic, SSRI, and/or antipsychotic) and found a higher percentage of genotypes with no functional alleles in the group of patients with EPS as compared to those without EPS. However, again, this was a mixed sample (and the EPS were various, including dystonia and akathisia). Scordo *et al.* (2000) investigated the association between EPS (dystonia, parkinsonism, or TD) and *CYP2D6* genotype (including duplications) in 119 patients with schizophrenia treated with antipsychotics, and found no difference in the distribution of homozygous EM, heterozygous EM, and UM between the 2 groups. However, all 4 PMs had a history of EPS. The authors concluded that PM genotype might be a predisposing factor for antipsychotic-induced EPS, but acknowledged the doubtful nature of this conclusion owing to the small number of PMs in the sample.

The failure to find a clear correlation between DIP and CYP2D6 PM status may firstly be due to the limited statistical power of the sample to detect a small effect size. The sample has a power of 0.93 (allelic comparison) or 0.68 (genotypic comparison) at the $P < 0.05$ level to detect a medium effect size (w 0.30), but the power drops to 0.21 (allelic) or 0.13 (genotypic) assuming an effect size of only 0.10. Similarly, the power of this sample for the logistic regression analysis is low (0.26, calculated using values of base proportion, i.e., percentage of cases with DIP = 0.53, OR = 1.39, N = 66, α = 0.05, and $R^2 = 0.0248$, NCSS 2000). Secondly, a significant association may have been

negated by clinical employment of the "neuroleptic threshold" (McEvoy, 1986), *i.e.*, titration of antipsychotic dose to the point where cogwheel rigidity discernible from baseline is detected. By this method, differential susceptibility to DIP would be obscured by dose titration, a higher incidence of DIP not being seen amongst CYP2D6 PMs in our sample due to lower doses being employed. In support of this hypothesis is the finding of Andreasson *et al.* (1997) of a non-significant tendency for PMs to have higher DIP ratings as compared to EMs, and the finding by Arthur *et al.* (1995) that patients with the highest DIP ratings tended to have the lowest neuroleptic doses. In both of these studies the high DIP ratings may reflect failure to titrate down the neuroleptic dose to a sufficient extent in CYP2D6 PMs. Unfortunately, for our 66 subjects rated for DIP, I know only that the typical antipsychotic dose was at least 100 mg chlorpromazine equivalents, but not the actual dose, and, furthermore, have no information regarding concomitant medications including anticholinergics. I do, however, know that the most common typical antipsychotic prescribed in this group was haloperidol (32% of cases), and that due to the complex involvement of CYP2D6 in haloperidol metabolism, the direction of effect of *CYP2D6* genotype on clinical response to haloperidol may be in the opposite direction to that with other antipsychotics metabolised by CYP2D6 (section 3.2.3.3). Furthermore, Spina *et al.* (1991) showed that phenothiazine antipsychotics were more potent inhibitors of CYP2D6 than haloperidol. It may therefore be that an association between relatively low CYP2D6 activity and antipsychotic adverse effects is additionally more likely to be found with typical antipsychotics other than haloperidol because of this inhibitory effect.

The figure of 18% for persistent TD is in agreement with values reported by other authors (Kane *et al.*, 1986; van Os *et al.*, 1997; Muscettola *et al.*, 1993). The finding of duration of antipsychotic treatment and DIP as risk factors for TD is also in accordance with other studies (Brandon *et al.*, 1971; Richardson and Craig 1982; Wolf *et al.*, 1983; Caliguiri *et al.*, 1991; Salz *et al.*, 1991; Muscettola *et al.* 1993; van Os *et al.*, 1997). Given that most studies report an increased risk of emergence or severity of TD with older age (Kane *et al.*, 1982a, 1982b; Kane *et al.*, 1986; Waddington *et al.*, 1990; Morgenstern and Glazer, 1993; Muscettola *et al.*, 1993; Jeste and Caligiuri, 1993; Jeste *et al.*, 1995; van Os *et al.*, 1997, Segman and Lerer, 2002), the failure in this study to show an association between age and TD is surprising. However, other studies have also failed to show this association (Chouinard *et al.*, 1986; Waddington *et al.*, 1995b). I also found no association between gender and persistent TD in the sample. Although many cross-sectional studies report a higher incidence of TD in females as opposed to males (Kane *et al.*, 1986; Yassa and Jeste, 1992), most prospective studies have not found an association with gender, apart from van Os *et al.*, 1997, who found an association with male sex.

I found a significant association between *CYP2D6* gene dosage and probable TD (P = 0.04), and a trend towards an association between gene dosage and total AIMS score of at least 6 for these cases (P = 0.06), a trend towards an association between gene dosage and persistent TD (P = 0.14), but no association between gene dosage and total AIMS score.

Arthur *et al.* (1995) studied 16 patients with TD, and found only one CYP2D6 PM (by genotype) in this group, and did not find a correlation between CYP2D6 phenotype and

AIMS score. Andreasson *et al.* (1997) genotyped 100 patients with schizophrenia for the *CYP2D6* alleles *CYP2D6*3-7*, and found no significant difference in the frequency of CYP2D6 PMs in those with and without TD. However, they found a non-significant tendency for CYP2D6 PMs to have more severe TD ratings, and a trend towards a higher frequency of the PM genotype in patients with persistent TD (P = 0.08). Kapitany *et al.* (1998) genotyped 45 patients with schizophrenia for the *CYP2D6* alleles *CYP2D6*3-5*, and the one PM did not have TD. However, a higher frequency of TD (81.3%) in individuals heterozygous for the non-functional alleles tested as compared with the frequency (46.4%) in those homozygous wild-type was found. In addition, Kapitany *et al.* (1998) found higher TD ratings in heterozygous individuals as compared to homozygous wild-type subjects. Ohmori *et al.* (1998) genotyped 100 Japanese patients with schizophrenia for the *CYP2D6*3*, *CYP2D6*4*, and *CYP2D6*10* alleles, and found a significant association between *CYP2D6*10* genotype and total AIMS score (P = 0.014) and a modest association with TD occurrence (P = 0.051). However, the same group (Ohmori *et al.*, 1999) did not find an association between *CYP2D6*2* genotype and either total AIMS score (P = 0.28) or TD occurrence (P = 0.10). They suggested that the reason for the difference between their findings for the *CYP2D6*10* and *CYP2D6*2* alleles could be that the *CYP2D6*2* allele codes for only slightly reduced CYP2D6 activity, while the *CYP2D6*10* allele codes for moderately reduced enzyme activity (Sachse *et al.*, 1997). In fact, the 31G>A ($Val_{11}Met$, exon 1) variant, which is in allelic disequilibrium with the mutations constituting the *CYP2D6*2* allele (Marez *et al.*, 1997) was overrepresented in *ultrarapid* metabolisers in the study by Løvlie and colleagues (2001), although functional studies have not supported the 31G>A substitution as being a cause of UM status (Allorge *et al.*, 2001). Furthermore, Lam *et al.* (2001) did not find an association between *CYP2D6*10*

genotype and TD in men, only finding an association in female patients with TD. The sample of Scordo *et al.* (2000) included 15 patients with TD; there was no difference between the mean AIMS scores between those homozygous EM's and those having at least one non-functional *CYP2D6* allele.

In the context of the above conflicting results, and the fact that the most commonly prescribed antipsychotic in the sample was haloperidol, my finding of a trend towards a *positive* association between the number of functional CYP2D6 genes and TD but lack of association with total AIMS score is not too surprising. If the positive association is valid, one possible explanation for this would be that the metabolism of the typical antipsychotics (especially haloperidol) by CYP2D6 produces metabolites (or parent drug) that are associated with increased risk of TD, individuals with a greater number of functional *CYP2D6* genes producing more of such active moeities. The pharmacodynamic effects of most metabolites of typical antipsychotics have not been established, but in the case of thioridazine, for example, CYP2D6 catalyses the formation of mesoridazine, which has antipsychotic activity. Authors who have not found an association between TD and CYP2D6 PM status have previously suggested that this may be explained by CYP2D6 PMs having a low level of lifetime exposure to antipsychotics metabolised by CYP2D6 due to severe intolerance (Kapitany *et al.*, 1998). Similarly, one other possible reason for the trend towards a positive association between the number of functional *CYP2D6* genes and TD is that the dose has been titrated initially to the "neuroleptic threshold", higher doses being employed for those with higher CYP2D6 activity. With multiple-dosing, especially at higher doses, the phenotype of those who are genotypically high in metaboliser status might become relatively low, especially if there is a delayed effect of the dose on the phenotype so

that a dose which is initially at the neuroleptic threshold exceeds the threshold at steady-state. The exceeding of the neuroleptic threshold at the steady-state in individuals who in the drug-naïve state had relatively high CYP2D6 activity and were therefore prescribed higher doses of antipsychotics might then be associated with the occurrence of TD. As already mentioned, information regarding the exact dose for the majority of the sample was unavailable. However, for the 6 subjects for which dose information was available, the mean antipsychotic dose was higher in the subjects with TD than those without, and the mean dose of anticholinergic medication was also higher. The latter finding is consistent with the results of previous investigators that indicate that anticholinergics may exacerbate TD in some cases (Kiloh *et al.*, 1973; Gerlach & Thorsen, 1976; Burnett *et al.*, 1980; Moore *et al.*, 1980; Greil *et al.*, 1984).

Another cytochrome P450, CYP1A2, has recently been implicated in TD (Basile *et al.*, 2000). However, more recent studies investigating a putative association between *CYP1A2* and TD have been negative (Schulze *et al.*, 2001; Tsapakis *et al.*, 2002a). Other factors, e.g. variation in the dopamine D3 receptor (Steen *et al.*, 1997; Basile *et al.*, 1999; Segman *et al.*, 1999), and the 5-HT_{2A} and 5-HT_{2C} receptors (Segman *et al.*, 2000; Segman *et al.*, 2001; Segman & Lerer, 2002); may also contribute to a vulnerability to TD, possibly in interaction with pharmacokinetic factors such as CYP2D6, CYP1A2, and CYP17 (Segman *et al.*, 2002).

In conclusion, this study indicates that CYP2D6 PM status or number of functional *CYP2D6* genes need not be, in isolation, a risk factor for intolerance to typical antipsychotics, or specifically drug-induced parkinsonism, or tardive dyskinesia. Differences between my findings and those of previous investigators may reflect the

relatively high percentage of cases prescribed haloperidol in my study and differences in prescribing practice. A study investigating all the candidate genes implicated so far in the genesis of tardive dyskinesia, performing a multivariate analysis, might prove fruitful.

3.3 Tricyclic antidepressants (TCAs) and CYP2D6 and CYP2C19

3.3.1 Introduction

3.3.1.1 Cytochromes and TCA metabolism

As early as 1967, it was observed that 11 patients on a standard dose of the TCA desipramine showed a 36-fold variation in the steady-state plasma concentrations achieved (Sjöqvist *et al.*, 1967), and the importance of genetic factors in determining the steady-state levels of the TCA nortriptyline was shown in twin and family studies (Alexanderson *et al.*, 1969, Åsberg *et al.*, 1971a, Alexanderson, 1973). It was subsequently shown that the steady-state concentrations of desipramine and nortriptyline were related to CYP2D6 activity (Bertilsson and Åberg-Wistedt, 1983, Nordin *et al.*, 1985), that the hydroxylation of the TCAs amitriptyline and clomipramine were also associated with polymorphic CYP2D6 activity (Balant-Gorgia *et al.*, 1982; Balant-Gorgia *et al.*, 1986), and that the 2-hydroxylation of imipramine cosegregated with CYP2D6 phenotype and genotype in a Danish study of the families of 18 CYP2D6 poor metabolisers (Madsen *et al.*, 1996). In Chinese, the *CYP2D6*10* allele is seen to affect plasma levels of nortriptyline in a dose-dependent manner, with heterozygous individuals showing higher nortriptyline concentrations than homozygous

wild type, and **10* homozygotes showing the highest concentrations (Yue *et al.*, 1998). Dalén *et al.* (1998) found a linear relationship between the clearance of nortriptyline and the log $MR_{debrisoquine}$ and the number of functional CYP2D6 genes (for values of the latter of 0, 1, 2, 3, and 13), and Morita *et al.* (2000) showed that the number of mutant *CYP2D6* alleles accounted for 41% of the variance in log (nortriptyline level corrected for dose and body weight).

Other studies have demonstrated the involvement of CYP2C19, CYP2C9, CYP3A4, and CYP1A2 in TCA metabolism (reviewed in Jann and Cohen, 2000). An early *in vitro* study by Lemoine *et al.* (1993), which did not include studies of CYP2C19 or CYP2C18, demonstrated the involvement of CYP1A2 and CYP3A4 in imipramine *N*-demethylation. Similarly, Schmider *et al.* (1995) demonstrated the involvement of CYP3A isoforms in amitriptyline demethylation, and Spina *et al.* (1997a) and Wang *et al.* (1997) in *in vivo* inhibition studies, showed that CYP3A4 played a major role in imipramine *N*-demethylation. However, Koyama *et al.* (1996) studied imipramine and desipramine concentrations in relation to CYP2C19 phenotype (S-mephenytoin hydroxylation status), which showed that the mean *N*-demethylation index was significantly less in the CYP2C19 poor metabolisers. The same group went on to perform a detailed *in vitro* study (Koyama *et al.*, 1997), using hepatic microsomes of varying CYP2C19 activity (as determined by *in vitro S*-mephenytoin 4'-hydroxylation) and recombinant CYPs, and demonstrated that imipramine *N*-demethylation was catalysed by CYP2C19 and CYP1A2 (high affinity and low affinity components respectively), imipramine 2-hydroxylation is mediated by CYP2D6 and CYP2C19 (high affinity and low affinity components respectively), and that in individuals deficient in CYP2C19, CYP1A2 and CYP2D6 play a major role in imipramine *N*-

demethylation and 2-hydroxylation respectively. Among the recombinant human CYPs, CYP2C19, 2C18, 2D6, 1A2, 3A4, and 2B6 in rank order catalysed the *N*-demethylation, whereas CYP2D6, 2C19, 1A2, 2C18, and 3A4 catalysed the 2-hydroxylation. The delineation of the high and low affinity components came from analysis of the K_m values. A later monoclonal antibody inhibition study by Yang and colleagues (1999) gave similar results, concluding that imipramine was metabolised to 2-hydroxyimipramine by 2C19 and 2D6, and to desipramine by 1A2, 2C18, 2C19, and 2D6, with the contributions of the isoforms to desipramine formation varying for 2C19 (13-50%), 1A2 (23-41%), and 3A4 (8-26%).

Olesen and Linnet (1997a; 1997b) showed that CYP2D6 is the major isoform involved in nortriptyline and amitriptyline 10-hydroxylation, with CYP2C19 and CYP1A2 being involved in the demethylation of nortriptyline (CYP2C19 having the highest demethylation capacity), and CYP2C19 being the major enzyme responsible for the demethylation of amitriptyline. They also concluded that the relative affinities and capacities of the CYPs would predict that at toxic doses of amitriptyline, CYP3A4 would play a dominant role in the metabolism. Consistent with the amitriptyline study was the work of Venkatakrishnan *et al.* (1998), using a combination of *in vitro* enzyme kinetic and chemical inhibition studies, which concluded that CYP2C19 (high affinity component) was the major amitriptyline *N*-demethylase at low (therapeutically relevant) amitriptyline concentrations, whereas CYP3A4 (low affinity component) was estimated to be more important at higher amitriptyline concentrations. Also consistent was the work of the same group (Venkatakrishnan *et al.*, 1999), demonstrating that the 10-hydroxylation of nortriptyline was conducted by CYP2D6 (high affinity component) and CYP3A4 (low affinity component), with the relative magnitude of the contribution

of the latter being only 20%. This indicates that CYP3A4 inhibition is unlikely to have a marked effect on nortriptyline hydroxylation; however, CYP3A4 may play a more significant role in CYP2D6 poor metabolisers or individuals prescribed drugs that induce CYP3A4. These studies were, however, in part contradicted by an *in vitro* inhibition study by Ghahramani *et al.* (1997), which concluded that CYPs 3A4, 2C9, and 2D6 mediated the *N*-demethylation of amitriptyline, and did not find a role for CYP2C19. An *in vitro* inhibition study of clomipramine concluded that its *N*-demethylation was mediated mainly by CYP1A2 and CYP3A4, and to a lesser extent by CYP2C19 (Wu *et al.*, 1998). An *in vivo* study of levels of trimipramine and its metabolites in CYP2D6-, CYP2C19-, and CYP3A4/5-phenotyped patients was consistent with the involvement of CYP2D6 in the 2-hydroxylation of trimipramine, and CYP2C19 in the demethylation (Eap *et al.*, 2000).

The above studies may be summarised as showing that CYP2D6 catalyses hydroxylation of the parent TCA and *N*-demethylated metabolite, with CYP2C19 contributing mainly to the *N*-demethylation of the parent TCA, with a low affinity contribution to the hydroxylation reactions (Figure 3.8).

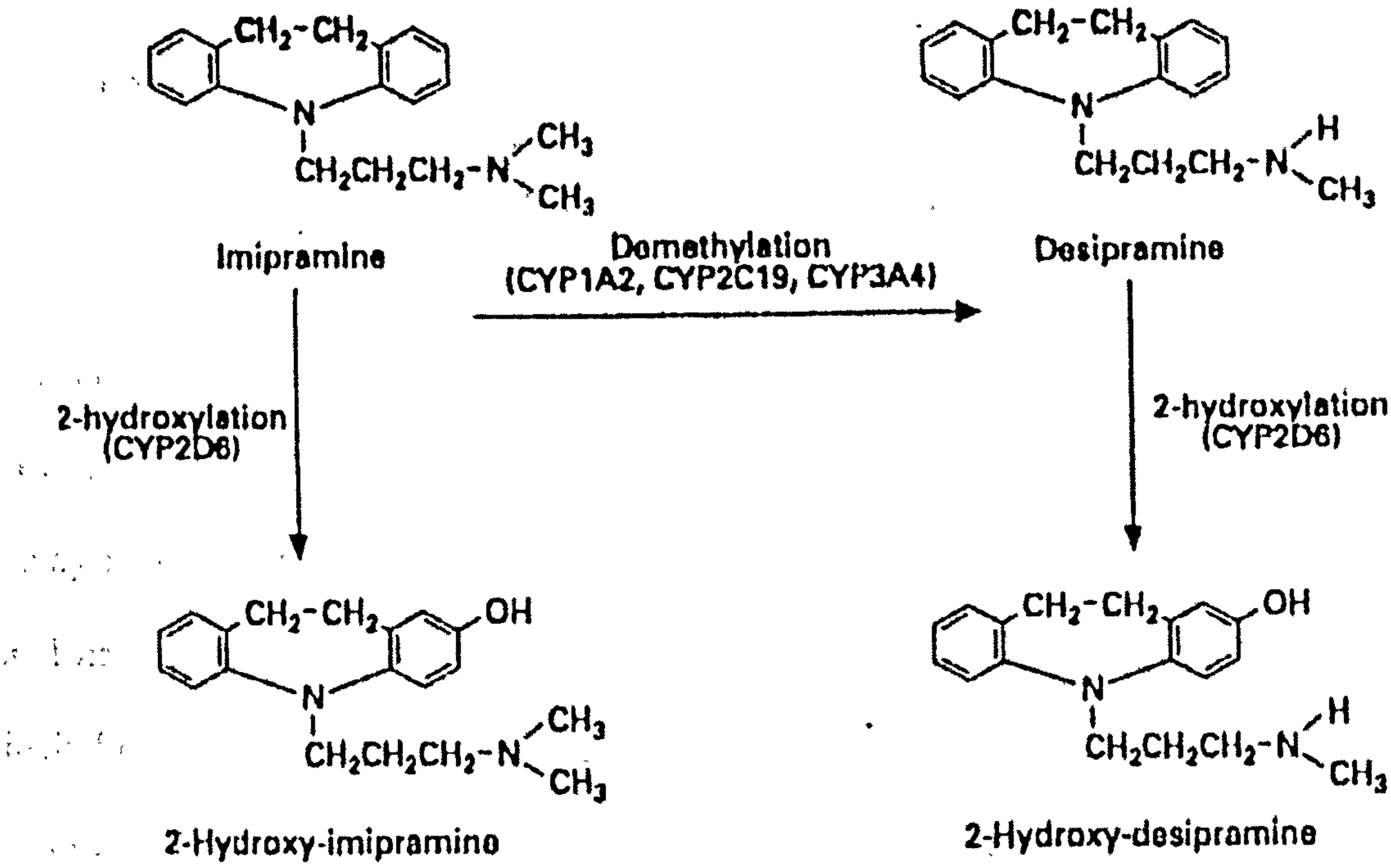


Figure 3.8 Initial steps in the metabolism of imipramine, with contributing cytochrome P450s shown (from Spina *et al.*, 1997b)

Several studies support the existence of a concentration-effect relationship for TCAs and/or their active metabolites (Ziegler *et al.*, 1977a; Sjöqvist *et al.*, 1980; Gram *et al.*, 1984; Perry *et al.*, 1987; Preskorn and Jerkovich, 1990). An early study linked high concentrations of nortriptyline with not only adverse effects, but also with decreased antidepressant effect (Åsberg *et al.*, 1971b). Subsequent reports have described concentration-dependent side-effects in individuals deficient in CYP2D6 treated with usual doses of TCAs due to the accumulation of the parent drug and/or active metabolites (Bertilsson *et al.*, 1981; Sjöqvist and Bertilsson, 1984; Balant-Gorgia *et al.*,

1989). There are interethnic differences in the distribution of various cytochrome P450 alleles (Aitchison *et al.*, 2000c), with, for example, Blacks showing a lower population mean CYP2D6 activity due to a high frequency of the *CYP2D6*17* allele (see Section 1.5.1.1; Masimirembwa *et al.*, 1996b; Droll *et al.*, 1998; Leathart *et al.*, 1998). It has been reported that at comparable doses of TCA, Black patients achieve higher levels of TCA than Caucasians, and also have a faster rate of recovery from a depressive episode (Raskin *et al.*, 1975; Ziegler *et al.*, 1977b; Rudorfer and Robins, 1982). A prospective study found that CYP2D6 poor metabolisers attained the highest levels of desipramine, and had adverse effects necessitating dose reduction (Spina *et al.*, 1997a). In a study including patients on TCAs, a trend towards an inverse correlation between the frequency of adverse drug events and number of functional CYP2D6 genes was found (Chou *et al.*, 2000).

Selective serotonin reuptake inhibitors, or SSRIs (*e.g.* paroxetine and fluoxetine, with paroxetine being a very potent inhibitor of CYP2D6), and the serotonin-noradrenaline reuptake inhibitor (SNRI) venlafaxine are also metabolised by CYP2D6 (Brøsen, 1993), and a higher frequency of inactivating *CYP2D6* alleles has been found amongst patients with a history of an adverse reaction to TCAs, relevant SSRIs, and venlafaxine (Chen *et al.*, 1996). Paroxetine is both a substrate for and an inhibitor of CYP2D6 (Bourin *et al.*, 2001), and Lam and colleagues (2002) described a subject with at least three functional *CYP2D6* genes who had an undetectable concentration of paroxetine. Fluoxetine is also a relatively potent inhibitor of CYP2D6, and because of its longer half-life, this inhibitory effect lasts much longer than for other other SSRIs with CYP2D6 inhibitory activity (Liston *et al.*, 2002). The major metabolite of fluoxetine,

norfluoxetine, is a CYP3A inhibitor (Caccia *et al.*, 1998). The major route of metabolism for citalopram is CYP2C19 (Sindrup *et al.*, 1993).

At the other extreme of the metabolic spectrum, case reports have indicated an association between therapeutic resistance to antidepressant treatment and CYP2D6 ultrarapid metaboliser status (Bertilsson *et al.*, 1993; Baumann *et al.*, 1998; Lam et al., 2002).

Cases of *combined* cytochrome P450 isoform deficiency may lead to particularly marked adverse effects: Bluhm *et al.* (1993) describe a case report of an individual who was a CYP2D6 poor metaboliser, and a CYP2C19 intermediate metaboliser who had had a normal baseline ECG, but developed myocardial ischaemia with doses of desipramine within the usual prescribed range. Most studies investigating correlations between antidepressant effects and metaboliser status have studied one, with few studying 2 cytochrome P450s, or controlling for the presence of concomitant medications affecting CYP metaboliser status.

3.3.1.2 Aims

The aims of this study involving patients treated with TCAs were to test the following hypotheses:

1. CYP2D6 ultrarapid metaboliser status is associated with low levels of parent TCA plus demethylated metabolite, corrected for dose, and therapeutic resistance to treatment.

2 Low CYP2D6 activity (due to the presence of at least one non-functional allele) is associated with high levels of parent TCA plus demethylated metabolite, and a high incidence of adverse effects.

3 Low CYP2C19 activity (due to the presence of at least one non-functional allele) is associated with high levels of the parent TCA, and low levels of the demethylated metabolite. This was hypothesised to result in a higher incidence of the effects consistent with the parent TCA, and a lower incidence of the effects consistent with the demethylated metabolite.

4 Items 1 and 2 may be summarised as: the number of functional *CYP2D6* genes is inversely associated with response to TCA and susceptibility to adverse effects of TCA.

5 The combination of relatively low CYP2D6 activity and low CYP2C19 activity was hypothesised to render an individual particularly susceptible to TCA adverse effects.

3.3.2 Methods

Details of the sample are given in Section 2.1.2.4. Preliminary analyses of this study have been reported (Aitchison *et al.*, 2001a; Tandon *et al.*, 2002b). Patients who consented and were not ineligible for phenotyping (*e.g.* concomitant antihypertensive treatment) were phenotyped for CYP2D6 status using the debrisoquine test (Lennard *et al.*, 1977). Genotyping for *CYP2D6* alleles **3-5* and gene duplication was undertaken as described (Aitchison *et al.*, 1999b, also sections 2.2.3.1 and 3.2.3.1). Genotyping for *CYP2C19* alleles **2* and **3* was undertaken as described by de Morais *et al.* (1994a and 1994b), with modifications to the protocol for **3* as described by Kubota *et al.* (1996), and employing a "hotstart" by the use of AmpliTaq Gold (Applied Biosystems). In

brief, for *CYP2C19*2*, primers 5'-AATTACAACCAGAGCTTGGC-3' and 5'-TATCACTTTCCATAAAAGCAAG-3' were used in a total reaction volume of 25 µl using Perkin-Elmer Buffer II (without magnesium), with 1.25 mM $MgCl_2$, 0.06 mM each dNTP, 0.25 µM each primer, and 1.5U AmpliTaq Gold (Perkin-Elmer, UK). Cycling conditions (on a MJ Research tetrad using 96-well plates) were: initial denaturation at 94^{0}C for 10 min (a long initial denaturation is required for AmpliTaq Gold); 38 cycles at 94^{0}C for 30 s, 58^{0}C for 30 s, and 72^{0}C for 1 min; followed by final elongation at 72^{0}C for 7 min. PCR products were digested using *Sma* I, and analysed on a 3% agarose gel. For *CYP2C19*3* assay, primers 5'-AACATCAGGATTGTAAGCAC-3' and 5'-TCAGGGCTTGGTCAATATAG-3' were used in a total reaction volume of 25 µl using Perkin-Elmer Buffer II (without magnesium), with 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.2 µM each primer, and 1.5U AmpliTaq Gold (Perkin-Elmer, UK). Cycling conditions (on a Perkin-Elmer Thermal Cycler using 0.5 ml eppendorf tubes) were: initial denaturation at 94^{0}C for 5 min; 40 cycles at 94^{0}C for 1 min, 57^{0}C for 1 min, and 72^{0}C for 2 min; followed by final elongation at 72^{0}C for 5 min. PCR products were digested using *Bam* HI, and analysed on a 3% agarose gel.

Association analysis, linear regression, and logistic regression analysis were conducted using EpiInfo version 6.0, and SPSS version 8.0, as described in sections 2.3.2 and 2.3.3. Graphs were plotted using Axum 5.0, and power was calculated with Solo Power Analysis. In addition, the combined effect of CYP2D6 and CYP2C19 activity was analysed by two different ways:

3. A simple model, assuming additive effect of number of CYP2D6 and CYP2C19 genes: creating a new variable by multiplying the number of *CYP2D6* and number of *CYP2C19* functional genes together, and analysing for association with clinical response, *etc.*

4. The creation of a variable (combined CYP2D6 and CYP2C19 index) approximating to the combined effect of these CYP2D6 and CYP2C19 activities as reflected in the serum level data: (the inverse of the dose corrected combined TCA level) x (the demethylation index). This was derived as follows:

CYP2D6 activity is inversely proportional to the ([parent TCA] plus [demethylated metabolite])/ dose = 1/dose corrected combined TCA level

CYP2C19 activity is proportional to the ratio of the [demethylated metabolite] to the [parent TCA] = demethylation index

Where [...] denotes "concentration of" in µg/l

Hence CYP2D6 x CYP2C19 activity is proportional to 1/dose corrected combined TCA level x demethylation index.

3.3.3 Sample characteristics

Out of the 49 individuals recruited, one was excluded as there was no clear history of affective disorder (the TCA was being prescribed as analgesia), and another was excluded due to inadequate clinical data being available. Of the remaining 47, forty had a diagnosis of unipolar depression, and 7 bipolar affective disorder. Five did not have both pre- and post-treatment Hamilton Depression Rating Scale (HDRS) scores, but an overall measure of response as judged by the treating clinician. Of the 42 with

pre- and post-treatment HDRS ratings, 11 were non-responders (non-responders being defined as individuals with an HDRS score of at least 16 after at least 6 weeks of TCA treatment, Danish University Antidepressant Group, 1999), 31 were responders, giving a response rate of 74%. The mean pre-treatment HDRS was 25.56 (SD 4.70, range 13-36); the mean post-treatment HDRS was 12.52 (SD 6.23, range 1-24). If the whole sample was included, including those with merely dichotomised response based on clinician opinion, there were 13 non-responders out of a total of 47, giving a response rate of 72%. Forty-two subjects had adverse effect scale ratings; the mean rating was 7.29 (SD 5.01, range 1-20). The mean 8-item corrected total adverse effect score (as in Ziegler *et al.*, 1978) was 4.88 (sd 3.35, range 0-13). Information on TCA level was available for 44 subjects; details of the TCA and concomitant prescribed medication for these 44 are given in Table 3.13. For details of CYP substrates, see Table 1.2. Binary variables for CYP2D6 and CYP2C19 inhibition were created, where 0 = no CYP inhibition, 1 = CYP inhibition by concomitant medication; Table 3.13 gives details of which medications were deemed to be CYP2D6 and/or CYP2C19 inhibitors.

The mean age of subjects was 44.38 (SD 11.14, range 25-69 years), twenty-five (53.2 per cent) were female, and twenty-four (51.1 per cent, smoking data was not available on one subject) were non-smokers. Thirty-seven subjects were Western European Caucasian, the rest were of varying ethnic origin (4 Mediterranean Caucasian, 1 mixed race, 1 Hispanic, 2 Indian, and 2 Afro-Caribbean). Thirty-four subjects consented to phenotyping with debrisoquine, and were not ineligible for phenotyping. For one individual, the phenotyping result was "no metabolite detected." This individual was therefore assigned a poor metaboliser phenotype, and for the purpose of linear regression was assigned an MR of 15.00 (above all the other MR values and in the poor

Tricyclic antidepressant (TCA)	Concomitant medication
Amitriptyline 150 mg	
Dothiepin 175 mg	
Dothiepin 75 mg	Chlorpromazine[1] 50 mg, 1.5 mg haloperidol[1] prn
Amitriptyline 225 mg	
Amitriptyline 200 mg	Trifluoperazine[1] 8 mg
Amitriptyline 75 mg	
Imipramine 300 mg	Diazepam[2] 10 mg
Amitriptyline 100 mg	Lithium 1 g, chlorpromazine[1] 50 mg, temazepam 40 mg
Lofepramine 70 mg	
Amitriptyline 100 mg	Lisinopril 2.5 mg, cimetidine[1,2] 800 mg, aspirin 75 mg
Amitriptyline 125 mg	Diazepam[2] 7.5 – 15 mg, diclofenac sodium[2] 150 mg, propranolol[1,2] 80 mg
Amitriptyline 200 mg	Ranitidine 600 mg
Imipramine 150 mg	Carbamazepine 400 mg
Lofepramine 140 mg	Lithium 600 mg, atenolol[1] 50 mg, thioridazine[1] 50 mg
Amitriptyline 100 mg	Insulin
Amitriptyline 100 mg	Paracetamol up to 6 mg daily, omeprazole[2] prn
Amitriptyline 100 mg	Lithium 600 mg
Imipramine 200 mg	Indapamide 2.5 mg, captopril 100 mg
Lofepramine 140 mg	
Amitriptyline 250 mg	Nifedipine 40 mg, thioridazine[1] 100 mg, codydramol[1] up to 4 tablets daily
Lofepramine 70 mg	
Imipramine 100 mg	Carbamazepine 400 mg
Imipramine 75 mg	
Amitriptyline 150 mg	
Amitriptyline 150 mg	
Trimipramine 150 mg	
Amitriptyline 200 mg	Fluoxetine[1] 20 mg, chlorpromazine[1] 50 mg, cocodamol[1] 4 tablets daily
Amitriptyline 100 mg	Chlorpromazine[1] 50 mg, lithium 1200 mg
Lofepramine 140 mg	Thyroxine 100 micrograms, zopiclone 7.5 mg
Imipramine 150 mg	
Nortriptyline 50 mg	
Amitriptyline 50 mg	
Trimipramine 150 mg	Haloperidol[1] 5 mg, lisinopril 5 mg
Trimipramine 100 mg	Haloperidol[1] 5 mg, lisinopril 5 mg
Trimipramine 150 mg	Zopiclone 7.5 mg
Trimipramine 300 mg	Indomethacin 75 mg, amlodipine 5 mg
Lofepramine 70 mg	
Trimipramine 125 mg	
Trimipramine 75 mg	
Trimipramine 150 mg	
Trimipramine 125 mg	
Trimipramine 75 mg	Cimetidine[1,2] 800 mg, diltiazem180 mg, captopril 50 mg, zimovane 7.5 mg
Trimipramine 125 mg	Zopiclone 7.5 mg, thioridazine[1] 50 mg every other day
Amitriptyline 150 mg	Thioridazine[1] 150 mg

Table 3.13 Details of tricyclic antidepressant (TCA) dose, and concomitant prescribed medications ([1]CYP2D6 inhibitors, [2]CYP2C19 inhibitors) for the 44 subjects for whom TCA level data were available

metaboliser range). Excluding this individual, the mean debrisoquine MR was 2.22 (SD 2.98, range 0.21-12.28).

3.3.4 CYP2D6 UM status and failure to respond to TCAs

No individuals who were positive for a CYP2D6 gene duplication/amplification event were found amongst the non-responders, or the sample as a whole.

3.3.5 Number of functional *CYP2D6* genes and clinical response to TCAs

The results of the linear regression analysis are given in Tables 3.14 and 3.15. Controlling for concomitant CYP2D6 inhibition, as expected, a significant association was found between debrisoquine MR and *CYP2D6* gene dosage ($P = 0.006$), and between the corrected combined TCA level and number of functional *CYP2D6* genes ($P = 0.001$, Figures 3.6 and 3.7). Controlling for CYP2D6 inhibition, there was a trend for an association between corrected combined TCA level and clinical response to TCA as measured by the percentage change in HDRS ($P = 0.08$). However, there was no association between percentage change in HDRS and *CYP2D6* gene dosage on linear regression, or between dichotomised response to TCA and *CYP2D6* gene dosage on logistic regression analysis ($P = 0.52$).

When the genotyping was extended (by Dr Kopal Tandon) to include the *CYP2D6*2* allele, this allowed delineation of results into the following genotypic categories: EM/EM, EM/IM, IM/IM, EM/PM, IM/PM, and PM/PM. Linear regression, controlling for CYP2D6 inhibition, gave a significant association between debrisoquine metabolic ratio and genotypic category ($P = 0.003$) and between corrected combined TCA level and genotypic category thus defined ($P = 0.004$, Figures 3.8 and 3.9).

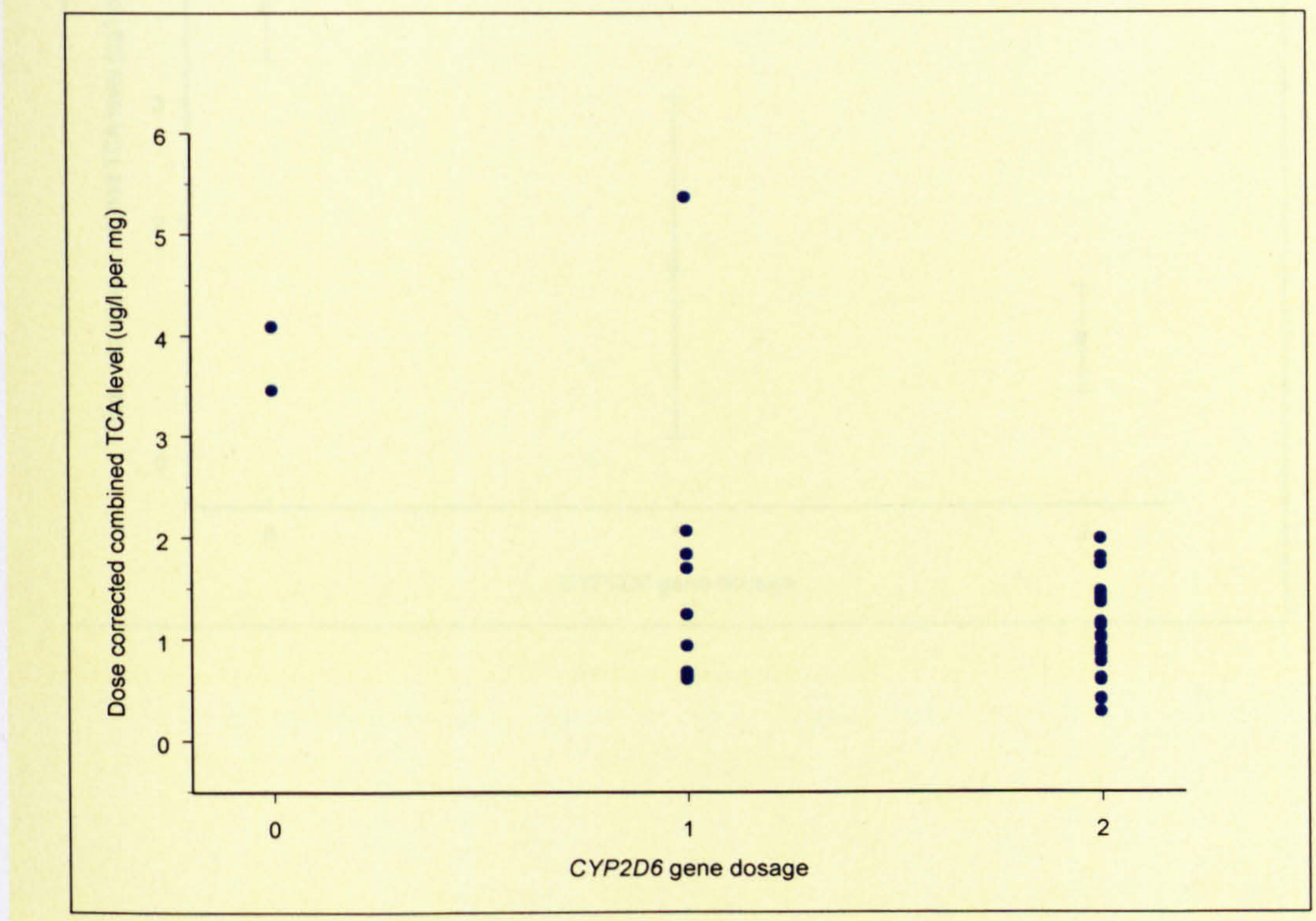


Figure 3.9 *CYP2D6* gene dosage (number of functional *CYP2D6* genes) versus dose corrected combined TCA level (μg/l per mg)

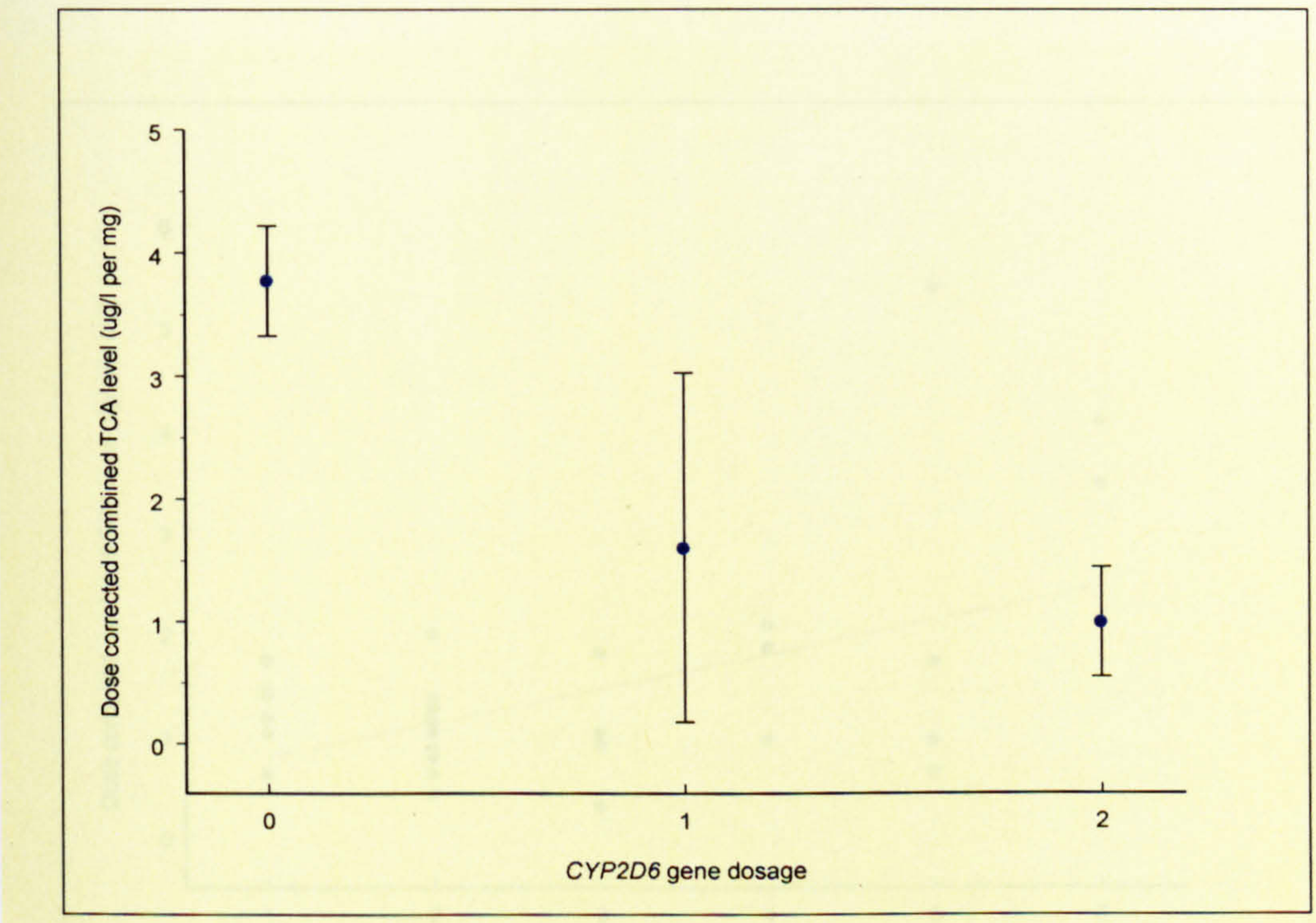


Figure 3.10 *CYP2D6* gene dosage (number of functional *CYP2D6* genes) versus dose corrected combined TCA level (μg/l per mg), mean values with standard deviations

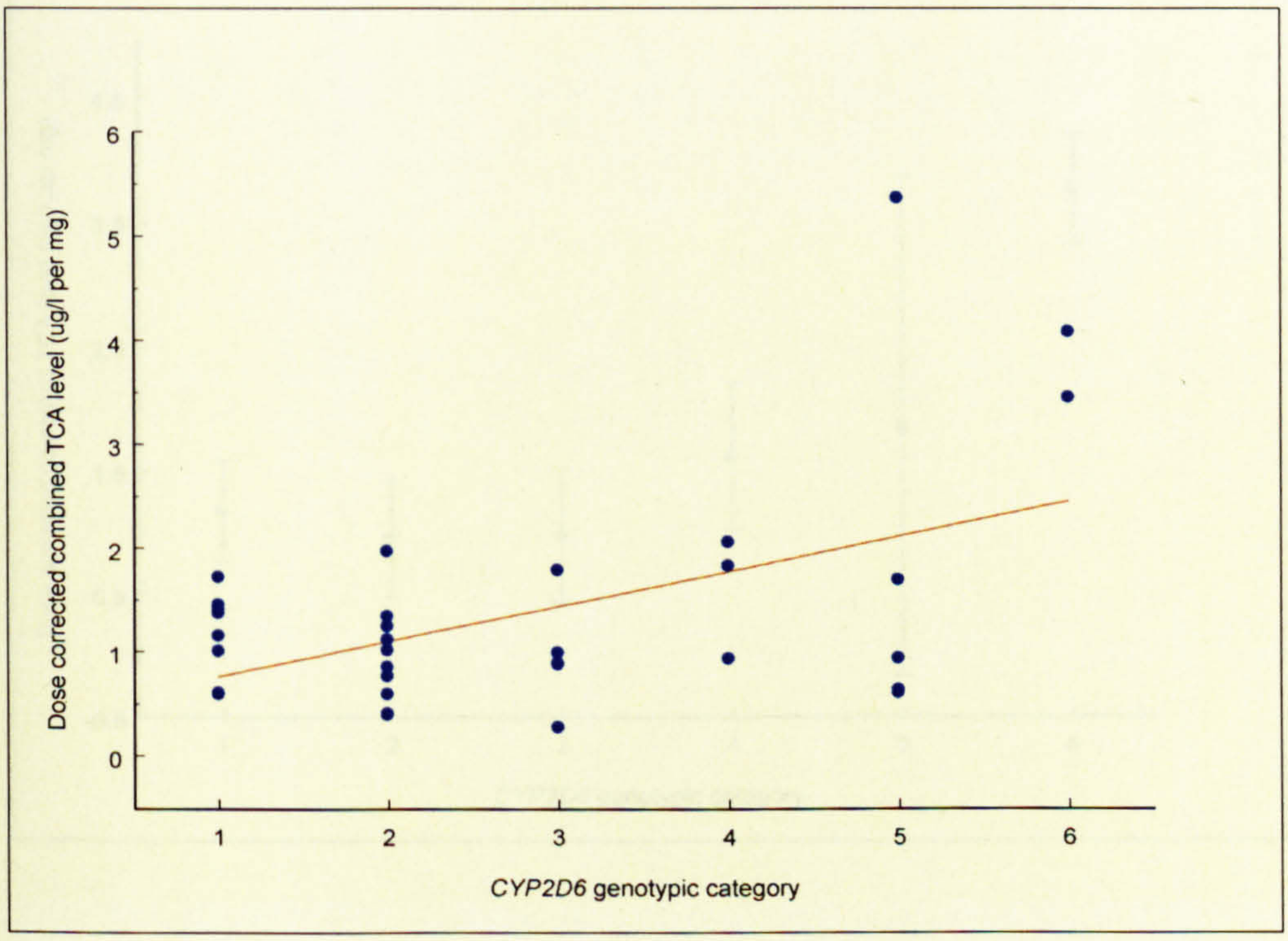


Figure 3.11 CYP2D6 genotypic category versus dose corrected combined TCA level (μg/l per mg). The linear regression line is shown. Genotypic categories are as follows: 1 = EM/EM, 2 = EM/IM, 3 = IM/IM, 4 = EM/PM, 5 = IM/PM, 6 = PM/PM.

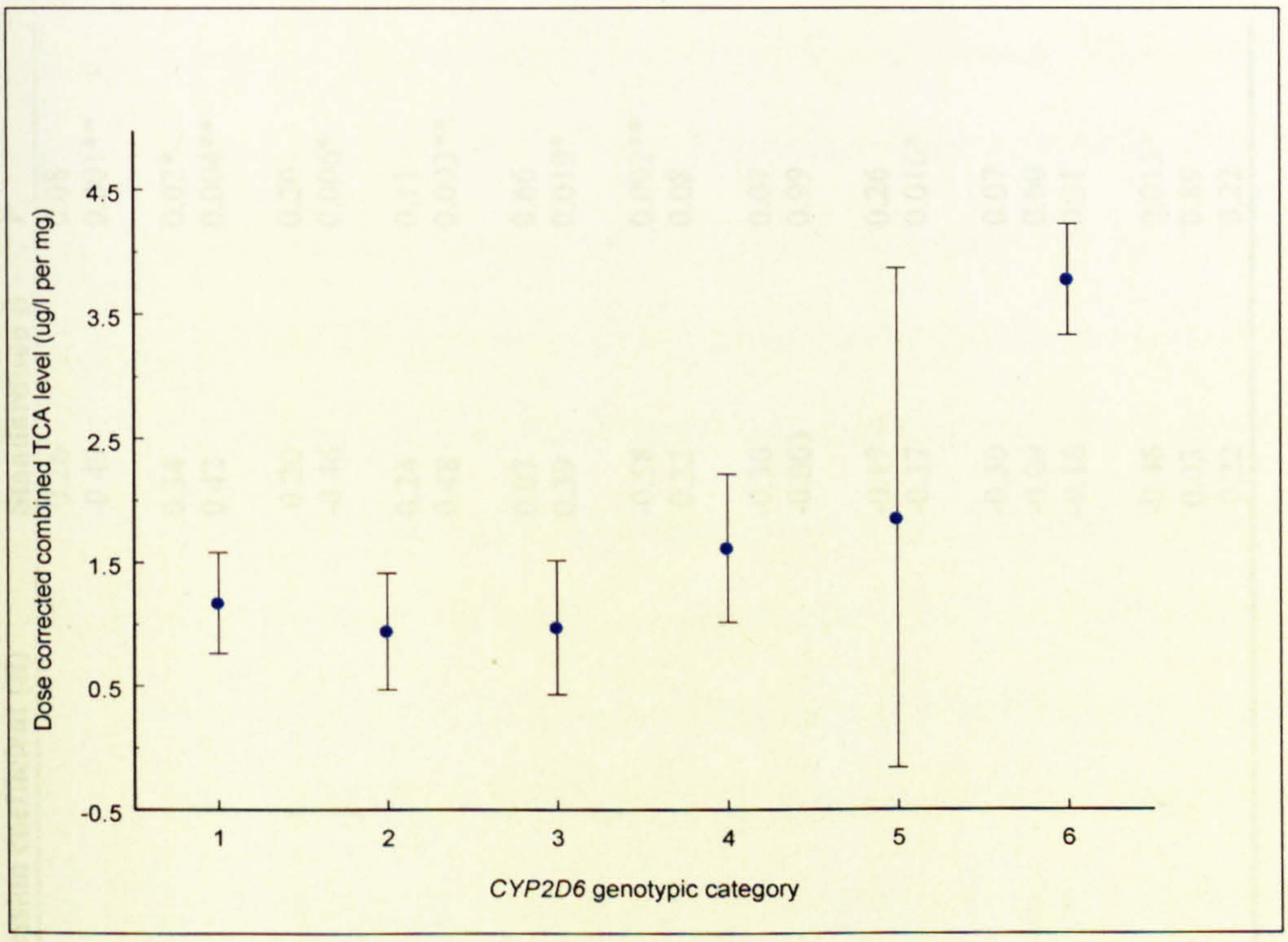


Figure 3.12 *CYP2D6* genotypic category (as in Figure 3.11) versus dose corrected combined TCA level (μg/l per mg), mean values with standard deviations shown

Table 3.14 Results of linear regression analysis for tricyclic antidepressant (TCA) study: clinical response

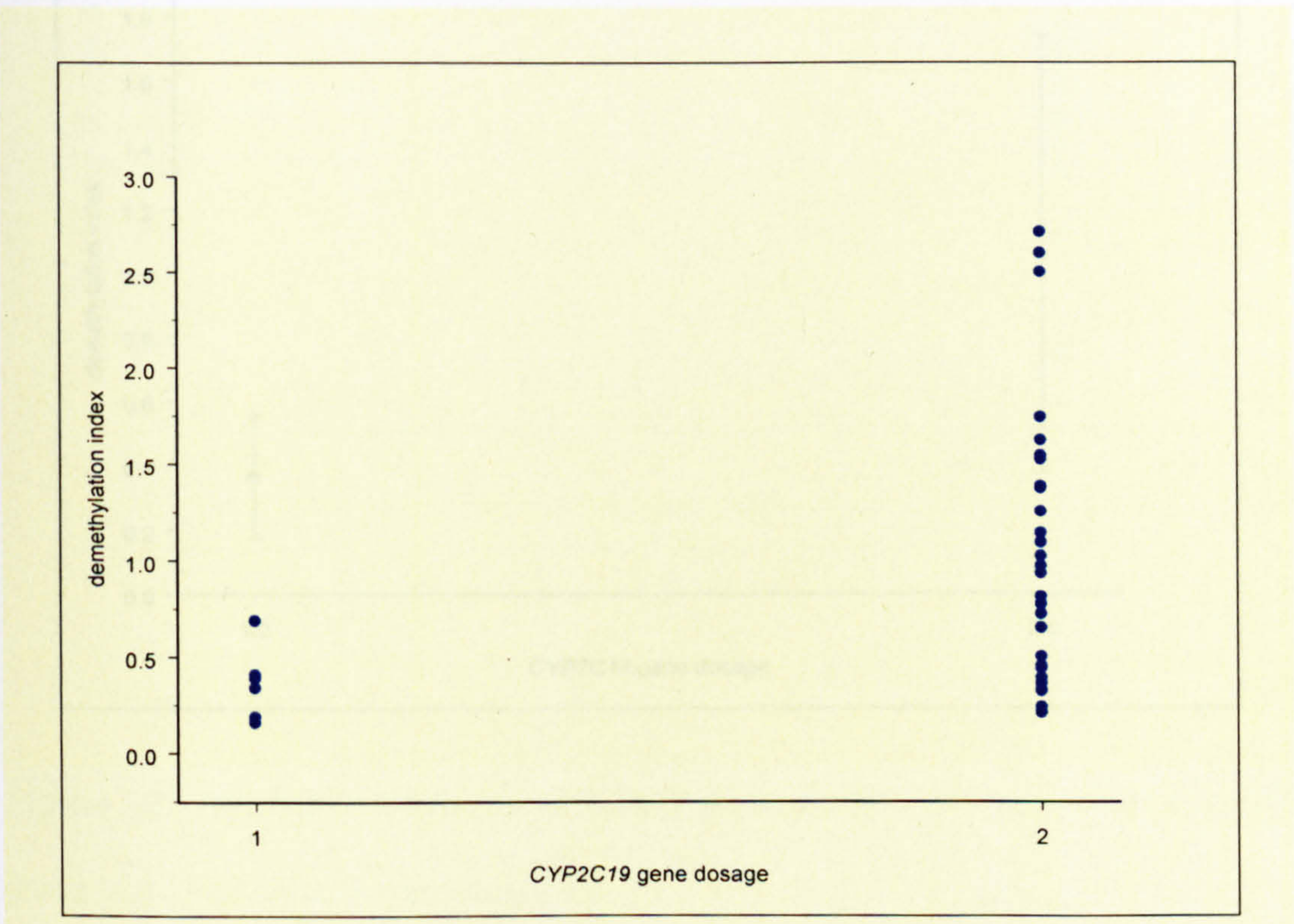
Dependent variable	Variables entered[1,2]	Regression coefficient (B)	Standardised β	*P*
Dose corrected combined TCA level	CYP2D6 inhibition[1]	0.52	0.26	0.08
	CYP2D6 gene dosage[2]	-0.85	-0.49	0.001**
Dose corrected combined TCA level	CYP2D6 inhibition[1]	0.69	0.34	0.02*
	CYP2D6 genotypic category[2]	0.27	0.42	0.004**
Debrisoquine metabolic ratio	CYP2D6 inhibition[1]	1.54	0.20	0.20
	CYP2D6 gene dosage[2]	-3.10	-0.46	0.006*
Debrisoquine metabolic ratio	CYP2D6 inhibition[1]	1.85	0.24	0.11
	CYP2D6 genotypic category[2]	1.23	0.48	0.003**
Demethylation index	CYP2C19 inhibition[1]	0.14	0.07	0.66
	CYP2C19 gene dosage[2]	0.70	0.39	0.019*
Percentage change in HDRS	CYP2D6 inhibition[1]	-26.4	-0.58	0.002**
	Dose corrected combined TCA level[2]	6.93	0.32	0.08
Percentage change in HDRS	CYP2D6 inhibition[1]	-14.3	-0.30	0.07
	CYP2D6 gene dosage[2]	-0.11	-0.003	0.99
Percentage change in HDRS	CYP2C19 inhibition[1]	-10.7	-0.17	0.26
	CYP2C19 gene dosage[2]	-20.5	-0.37	0.016*
Percentage change in HDRS	CYP2C19 inhibition[1]	-14.3	-0.30	0.07
	CYP2D6 inhibition[1]	-5.37	-0.09	0.60
	CYP2D62C19 gene dosage[2]	-2.93	-0.16	0.31
Percentage change in HDRS	CYP2C19 inhibition[1]	-20.0	-0.46	0.015*
	CYP2D6 inhibition[1]	1.49	0.03	0.89
	Combined CYP2D6 and CYP2C19 index[2]	-2.98	-0.22	0.22

In Figures 3.6 - 3.9 it can be clearly seen that there is an outlier in the IM/PM genotypic category in terms of corresponding dose corrected combined TCA level. This individual has been genotyped as **2/*4*, and has since been genotyped as *CYP2D6*6* negative. She was taking cimetidine 400 mg twice daily, amoxycillin 500 mg three times daily, captopril 25 mg twice daily, diltiazem 60 mg three times daily, and zimovane 7.5 mg daily at the time that the blood sample for TCA level was taken, in addition to the tricyclic medication (trimipramine 75 mg). The raised dose-corrected combined TCA level may therefore be due to the effect of CYP inhibition (by cimetidine and diltiazem, British National Formulary, September 2002) on top of the effect of the low CYP2D6 activity (**2* only), or, alternatively, this individual may have an inactivating mutation in *CYP2D6* not yet assayed for. DNA from this individual is being subjected to mutation screening using DHPLC (O'Donovan *et al.*, 1998). Unfortunately, this individual did not consent to phenotyping with debrisoquine.

3.3.6 Number of functional *CYP2C19* genes and clinical response to TCAs

No *CYP2C19* poor metabolisers were found in this sample and the number of functional *CYP2C19* genes is therefore only 1 or 2. Controlling for concomitant CYP2C19 inhibition, as expected, a significant association was found between the *N*-demethylation index and the number of functional *CYP2C19* genes ($P = 0.019$, Table 3.14, Figures 3.10 and 3.11). However, a novel finding was that controlling for CYP2C19 inhibition, there was a significant association between number of functional *CYP2C19* genes and percentage change in HDRS ($P = 0.016$). This is shown graphically in Figures 3.12 and 3.13. Of note, in the graphical representation, it is not possible to control for CYP2C19 inhibition, and therefore the association is less clear.

Figure 3.13 *CYP2C19* gene dosage (number of functional *CYP2C19* genes) versus demethylation index (ratio of demethylated metabolite to parent TCA)



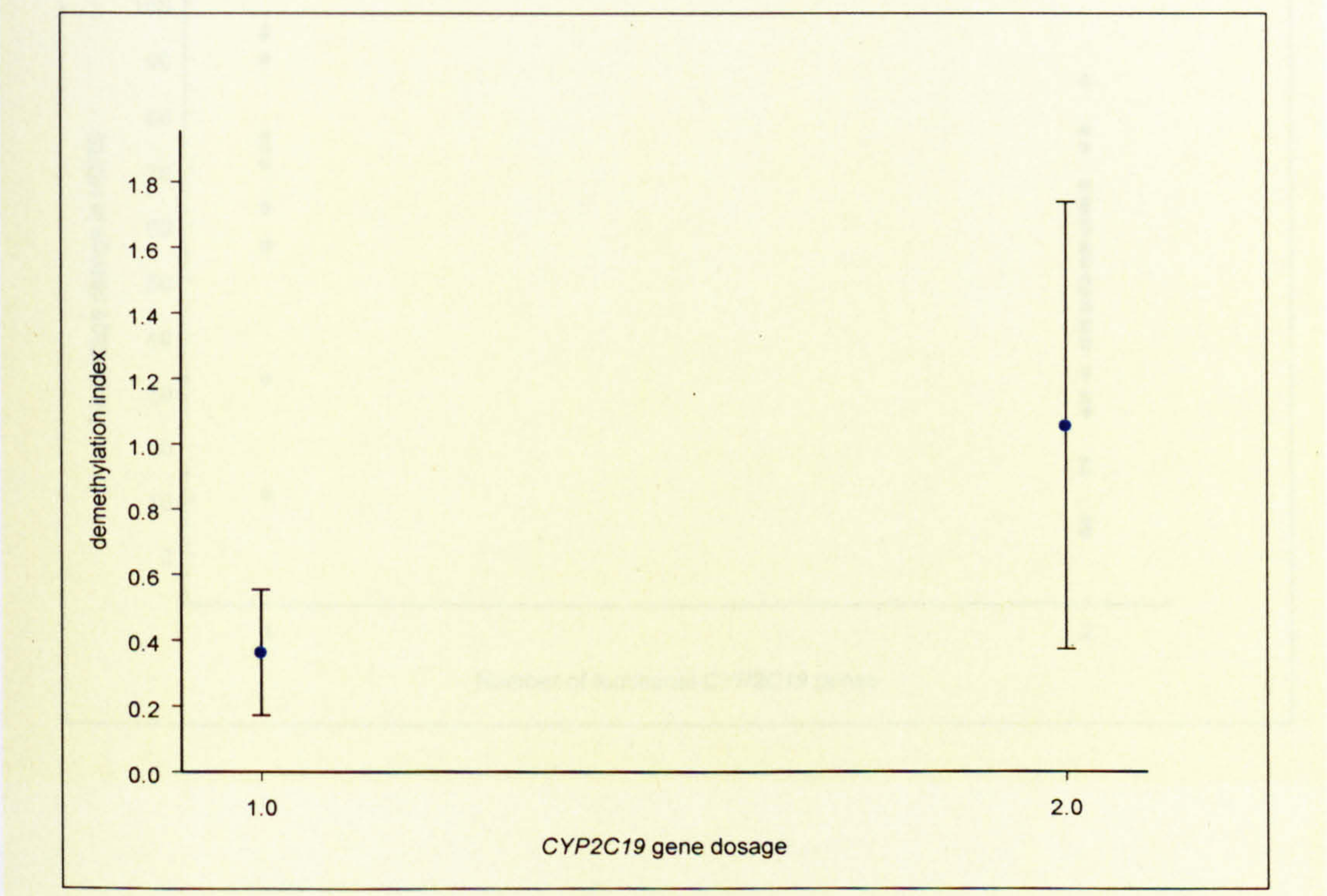


Figure 3.14 *CYP2C19* gene dosage (number of functional *CYP2C19* genes) versus demethylation index (ratio of demethylated metabolite to parent TCA), mean values with standard deviations given

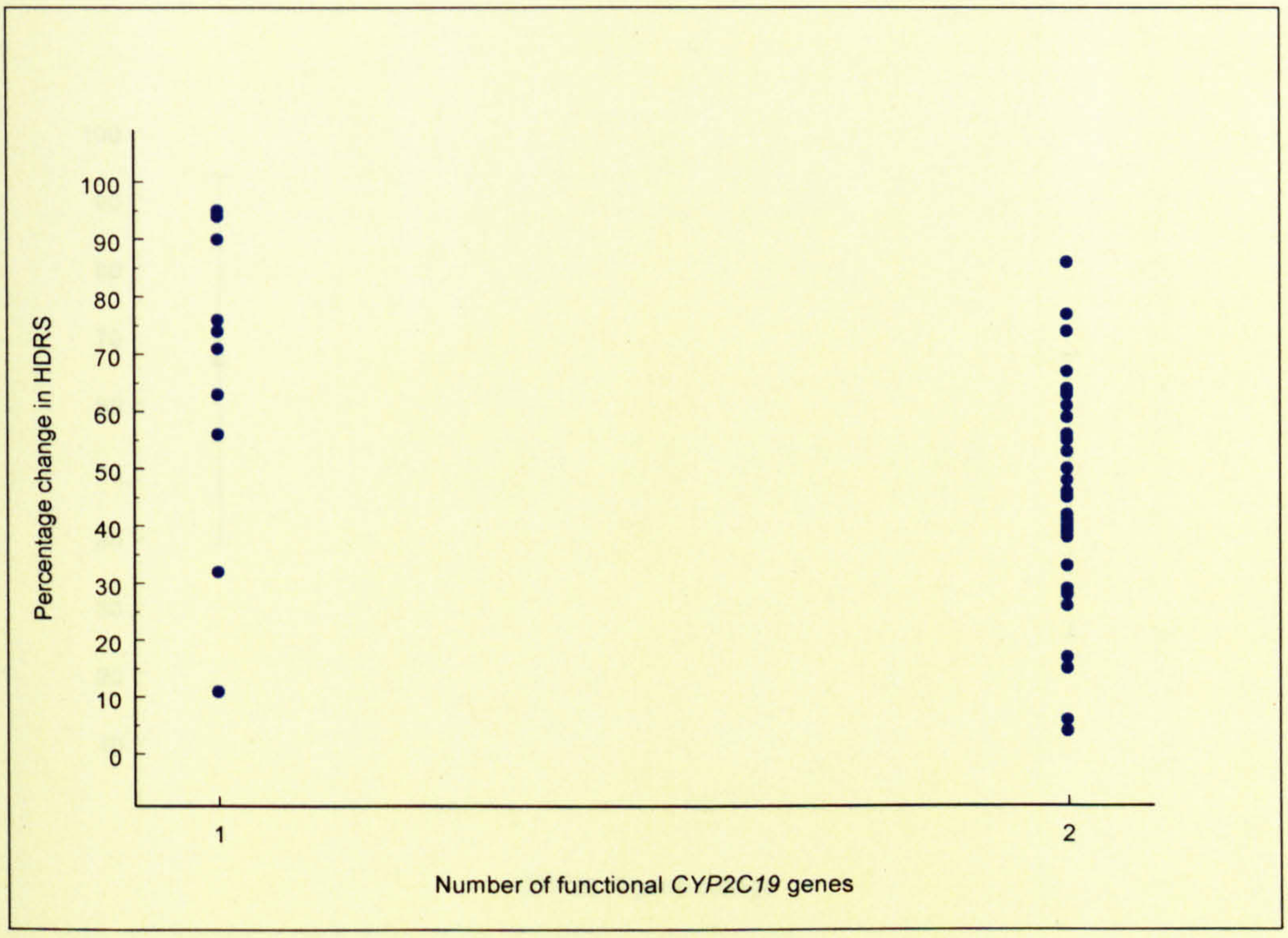


Figure 3.15 Number of functional *CYP2C19* genes versus percentage change in HDRS (Hamilton Depression Rating Scale)

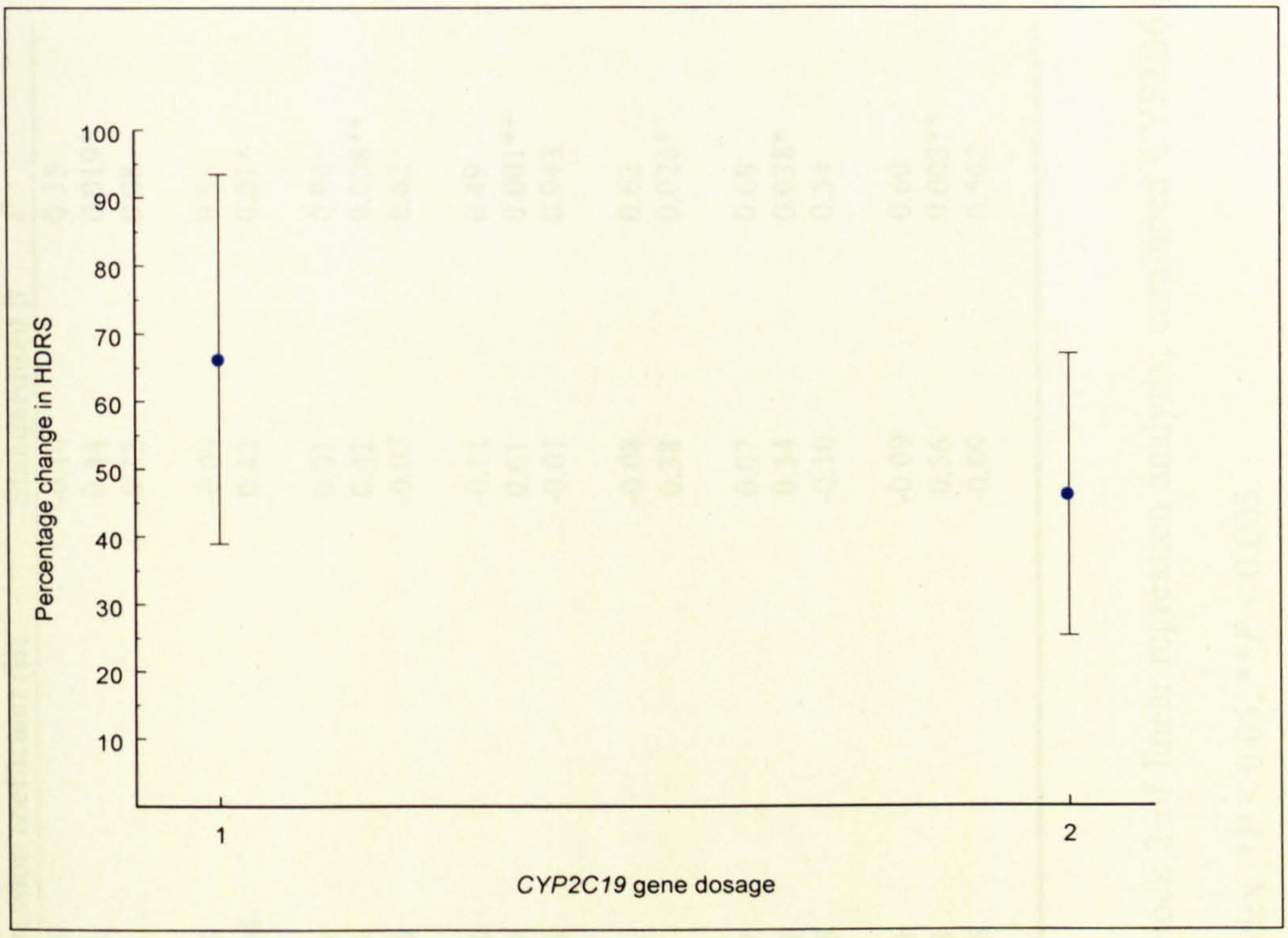


Figure 3.16 Number of functional *CYP2C19* genes (*CYP2C19* gene dosage) versus percentage change in HDRS (means with standard deviations given)

3.3.7 Associations with adverse effects

The associations between the total adverse effect score and the 8-item adverse effect score (as in Ziegler *et al.*, 1978) and clinical and genotypic parameters are given in Table 3.15. For the total adverse effect score, an association was found between adverse effect score and presence of concomitant CYP2D6 inhibiting medication (P = 0.019), and with the demethylated metabolite level (P = 0.01), and there was a trend for an association with the combined TCA level (P = 0.08). These effects were less marked for the 8-item adverse effect score (P = 0.026 versus demethylated metabolite).

Table 3.15 Results of linear regression analysis for tricyclic antidepressant (TCA) study: adverse effects

Dependent variable	Variables entered[1,2]	Regression coefficient (B)	Standardised β	*P*
Total adverse effect score	CYP2C19 inhibition[1]	-1.85	-0.14	0.35
	CYP2D6 inhibition[1]	4.11	0.44	0.019*
	Combined TCA level[2]	0.01	0.32	0.08
Total adverse effect score	CYP2C19 inhibition[1]	-1.25	-0.09	0.55
	Level of demethylated metabolite[2]	0.004	0.42	0.01*
Total adverse effect score	CYP2C19 inhibition[1]	0.14	0.01	0.94
	CYP2D6 inhibition[1]	4.15	0.42	0.008**
	CYP2D62C19 gene dosage[2]	-0.28	-0.07	0.62
Total adverse effect score	CYP2C19 inhibition[1]	-1.44	-0.11	0.49
	CYP2D6 inhibition[1]	5.69	0.61	0.001**
	Combined CYP2D6 and CYP2C19 index[2]	-0.03	-0.01	0.943
Ziegler 8-item adverse effect score	CYP2C19 inhibition[1]	0.70	-0.08	0.62
	Level of demethylated metabolite[2]	0.02	0.38	0.026*
Ziegler 8-item adverse effect score	CYP2C19 inhibition[1]	0.57	0.07	0.68
	CYP2D6 inhibition[1]	2.28	0.34	0.038*
	CYP2D62C19 gene dosage[2]	-0.25	-0.10	0.54
Ziegler 8-item adverse effect score	CYP2C19 inhibition[1]	-0.71	-0.09	0.60
	CYP2D6 inhibition[1]	3.28	0.56	0.003**
	Combined CYP2D6 and CYP2C19 index[2]	-0.36	-0.09	0.562

For Tables 3.14 and 3.15: [1]Variables entered in block 1, [2] variables entered in block 2 of linear regression analysis; combined CYP2D6 and CYP2C19 index = inverse dose corrected combined TCA level x demethylation index. *$P < 0.05$, **$P < 0.005$.

The association between the demethylated metabolite level and the total adverse effect score is shown graphically in Figure 3.17; again, without controlling for CYP2C19 inhibition, the association is less clear.

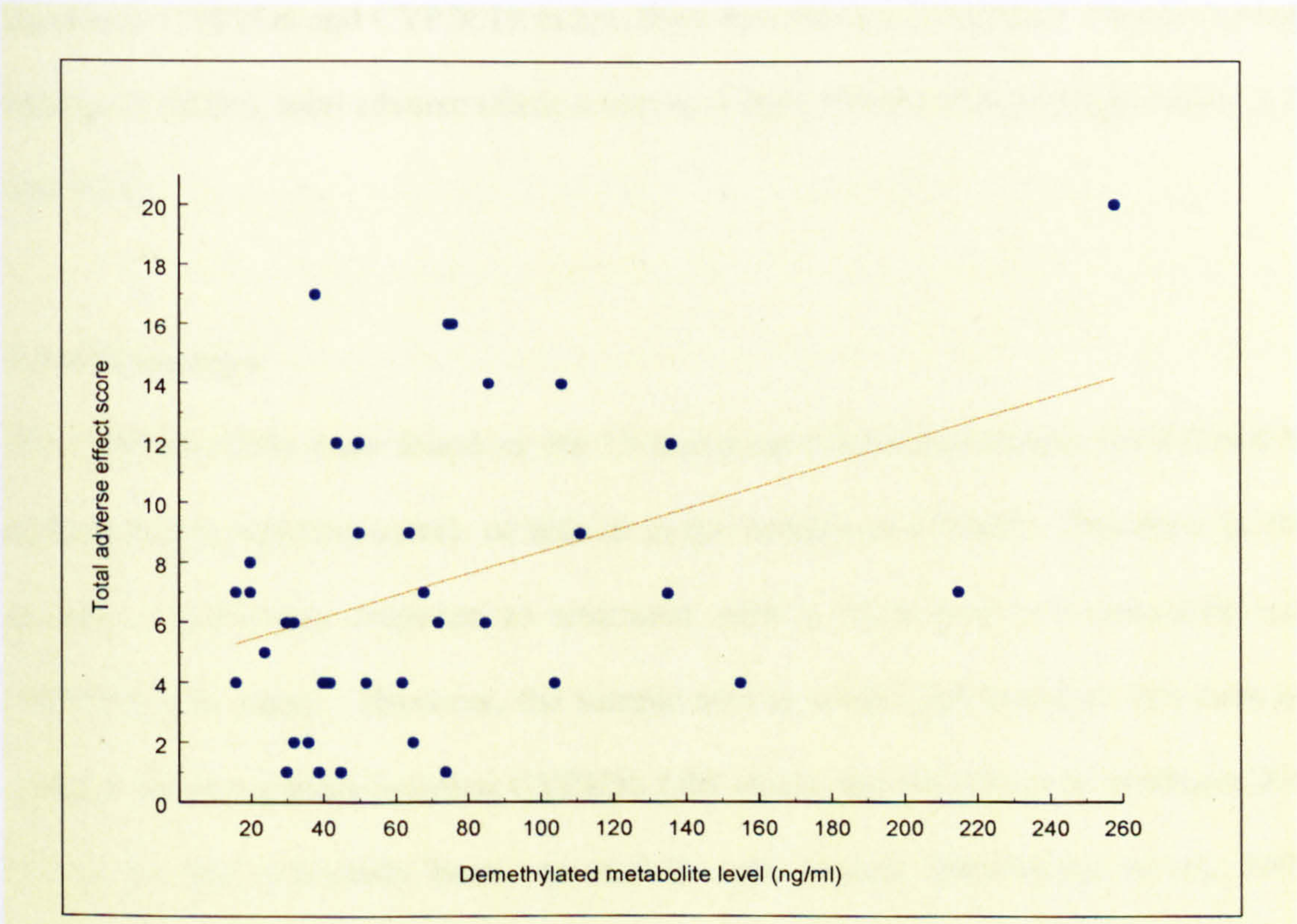


Figure 3.17 Demethylated metabolite level versus total adverse effect score, linear regression line (without controlling for CYP2C19 inhibition) shown

3.3.8 Investigation of possible interaction between number of functional *CYP2D6* genes and number of functional *CYP2C19* genes

The mean values of the combined CYP2D6 and CYP2C19 index in responders and non-responders were 1.28 (sd 1.76) and 0.84 (sd 0.42). As the variances were not equal

(Levene's test for equality of variances, $P = 0.114$), a nonparametric test (Mann-Whitney U test) was used to compare the values for the combined CYP2D6 and CYP2C19 index in responders and non-responders, which gave a non-significant result ($P = 1.0$). Using linear regression with either the simple additive model or the combined CYP2D6 and CYP2C19 index, there was also no association with percentage change in HDRS, total adverse effect score, or 8-item adverse effect rating (Tables 3.14 and 3.15).

3.3.9 Discussion

No CYP2D6 UMs were found in the 13 treatment-refractory subjects (as defined by dichotomised response score), or indeed in the sample as a whole. Therefore, in this sample, a refractory response to treatment with a TCA was not associated with CYP2D6 UM status. However, the sample size is small, and therefore this does not exclude an association between CYP2D6 UM status and resistance to treatment with TCAs, as has previously been reported in case reports (Bertilssson *et al.*, 1993; Baumann *et al.*, 1998). A larger prospective study is indicated to answer this question.

The second hypothesis tested was that low CYP2D6 activity would be associated with high levels of parent TCA plus demethylated metabolite, and hence a high incidence of adverse effects. A significant inverse association was indeed found between number of functional CYP2D6 genes and dose corrected combined TCA level ($P = 0.001$, Figures 3.6 and 3.7). A trend for an association was found between combined TCA level and total adverse effect score, controlling for CYP2D6 and CYP2C19 inhibition ($P = 0.08$), and between corrected combined TCA level and clinical response to TCA as measured by the percentage change in HDRS ($P = 0.08$). No association was found between

CYP2D6 gene dosage and total side effect score ($P = 0.59$), or between *CYP2D6* gene dosage and antidepressant effect ($P = 0.99$). The failure to find a significant association here may be due to the sample size; a power calculation of multiple regression analysis shows that for the power to reach at least 0.80, the additional R^2 to be contributed by the variable of interest must be at least 0.17 (1 variable of interest, 2 variables to be controlled for in the case of combined TCA level, 1 variable to be controlled for in the case of *CYP2D6* gene dosage, if additional R^2 contributed by the variables to be controlled is set at 0.02, sample size of 42, significance level of 0.05). For the analysis of combined TCA level, the R^2 change contributed by the variable of interest (from the linear regression output) is 0.07, the R^2 change contributed by the two variables controlled for (CYP2D6 and CYP2C19 inhibition) is 0.34, which gives a power of 0.56.

This is not the first study that has failed to find an association with CYP2D6 indices and antidepressant effect. In the study by Spina *et al.* (1997a), no significant correlation was found between plasma desipramine levels, or CYP2D6 phenotype as assessed by dextromethorphan metabolic ratio, and antidepressant effect. Moreover, in a retrospective study of patients taking imipramine, no correlation was found between CYP2D6 or CYP2C19 phenotype and frequency or severity of side effects (Meyer *et al.*, 1988), a small prospective study regarding imipramine treatment also found no correlation between CYP2C19 genotype and improvement rate or severity of side-effects (Morinobu *et al.*, 1997), and in their single-dose study administering nortriptyline, Dalen *et al.* (1998) found no correlation between severity of autonomic effects and number of functional CYP2D6 genes. One possible reason for the above negative results is the fact that for the hydroxylation reactions, CYP2D6 is high-affinity

low-capacity enzyme, with saturable kinetics (Brøsen *et al.*, 1986; Sindrup *et al.*, 1992; Danish University Antidepressant Group, 1999; Aitchison *et al.*, 2000c), and that with multiple-dosing, or at steady-state, CYP3A4, which is the most abundant hepatic cytochrome P450 (Shimada *et al.*, 1994), may play a more major role (Olesen and Linnet, 1997b). Furthermore, in enzyme deficiency, the cytochromes may substitute for each other (Koyama *et al.*, 1997; Venkatakrishnan *et al.*, 1999).

The third hypothesis was that low CYP2C19 activity would be associated with high levels of the parent TCA, low levels of the demethylated metabolite, and a consequent higher incidence of effects consistent with this combination. A significant association ($P = 0.019$, Figures 3.10 and 3.11) was indeed found between the number of functional *CYP2C19* genes and demethylation index (ratio of demethylated metabolite to parent TCA). Interestingly, a significant association was also found between *CYP2C19* gene dosage and percentage change in HDRS ($P = 0.016$), with the direction of effect being that two functional *CYP2C19* genes was associated with a poorer mean response than one functional *CYP2C19* gene. This suggests that the parent TCA is more important in terms of generating clinical response than the demethylated metabolite. On the other hand, a significant association was also found between the level of demethylated metabolite and total adverse effect score ($P = 0.01$), suggesting that the demethylated metabolite is more important than the parent TCA in the generation of adverse effects. The demethylated metabolite in general has a relatively high noradrenaline transporter affinity (see below), which might be expected to be associated with adverse effects such as orthostatic hypotension and palpitations. (The sample size was too small to perform an analysis of a subdivision of the adverse effect score versus CYP2C19 indices other than the 8-item score as in Ziegler *et al.*, 1978.) Interestingly, a novel

cytochrome found in rat brain and termed CYP2D18 is also capable of catalysing the demethylation of imipramine to desipramine (Kawashima *et al.*, 1996; Thompson *et al.*, 1998); it may be that the associations with CYP2C19 indices reflect local neuronal metabolism rather than or as well as peripheral hepatic metabolism.

The final hypothesis was that the combination of low CYP2D6 activity and low CYP2C19 activity would render an individual particularly susceptible to TCA adverse effects. Analysis of the combined effect of CYP2D6 and CYP2C19 gene dosage did not reveal any association with total adverse effect or 8-item adverse effect score; the only variable that emerged as being significantly associated with adverse effect score in this analysis was CYP2D6 inhibition (by concomitant medication).

Of note, the sample size for this study was small, and the *P* values have not been corrected for multiple testing. Using the Bonferonni correction for multiple testing, *P* values less than 0.005 would be significant (as approximately 10 separate association analyses were conducted, not counting the genotypic category analysis as separate from that with *CYP2D6* gene dosage, or the Ziegler 8-item side effect scale analysis as separate from total side effect score). On this basis, there would be significant associations between *CYP2D6* gene dosage and dose corrected combined TCA level, between *CYP2D6* gene dosage and debrisoquine MR, between CYP2D6 inhibition and percentage change in HDRS, and between CYP2D6 inhibition and total side effect score (Tables 3.14 and 3.15). The latter two associations would suggest that higher levels of parent TCA plus demethylated metabolite are associated with both clinical response and adverse effects.

The parent TCA and demethylated metabolites differ in their affinities for the serotonin and noradrenaline transporters (Owens, 1997), in general the demethylated metabolite having a higher noradrenaline to serotonin transporter affinity ratio. As there are antidepressants in clinical use that are either predominantly serotonin reuptake inhibiting (e.g. citalopram) or almost exclusively noradrenaline reuptake inhibiting (e.g. reboxetine), inhibition of either transporter may be associated with antidepressant effect. Furthermore, the serotoninergic and adrenergic pathways are interconnected, such that action on one pathway may influence the activity of the other (Ressler & Nemeroff, 1999). However, there is some evidence that especially in the case of patients hospitalised for depression, the use of an antidepressant with combined serotonin and noradrenaline reuptake inhibition (so-called dual reuptake inhibitors, including the tertiary amine tricyclics and venlafaxine) may be more efficacious than employing an agent that has a high affinity for one transporter (Clerc *et al.*, 1994; Leonard, 1999; Anderson, 2000; Thase *et al.*, 2001; Nolen & Bruijn, 2002). The association between CYP2D6 inhibition and clinical response would be consistent with this.

In conclusion, owing to the small sample size, no firm conclusions may be drawn between CYP2D6 or CYP2C19 gene dosage and clinical response as measured by HDRS or adverse effect ratings. However, the association with CYP2D6 inhibition, and the suggestions of associations between *CYP2C19* gene dosage and clinical response and between demethylated metabolite level and adverse effect rating are interesting findings and warrant replication in a larger prospective study

3.4 Response to clozapine and CYP1A2

3.4.1 Introduction

The atypical antipsychotic clozapine is the drug of choice in the UK for cases of treatment-resistant schizophrenia or schizoaffective disorder, and is effective in 30 to 60% of patients unresponsive to typical antipsychotics (Kane, 1992). It is metabolised mainly in the liver by demethylation and *N*-oxidation (Jann *et al.*, 1993). Several cytochrome P450 enzymes including CYP1A2 have been implicated in these processes (Eiermann *et al.*, 1997; Linnet and Olesen, 1997; Tugnait *et al.*, 1999). Jerling *et al.* (1997) suggested that, since the clearance of clozapine is distributed in a similar way to indices of CYP1A2 activity, CYP1A2 could be the major determinant of clozapine clearance; this was confirmed in my study with CYP1A2-null mice (Chapter 5; Aitchison *et al.*, 2000b), which was performed prior to this genetic association study.

There is wide interindividual variation in CYP1A2 activity (Aitchison *et al.*, 2000c), which in most studies is trimodally or bimodally distributed, indicating a likely genetic contribution to the variation in activity (Sections 1.5.3.1 and 4.6). In addition, CYP1A2 is subject to inhibition and induction by various agents, inducers including cigarette smoking. Several polymorphisms in *CYP1A2* have been identified, including SNPs in the 5' flanking region, a $G_{-3858}A$ substitution (*CYP1A2*1C*, Nakajima *et al.*, 1999), a $T_{-3591}G$ substitution, a $G_{-3595}T$ substitution, and a T_{-3605} insertion (Chapter 4; Aitchison *et al.*, 2000a); and a SNP in intron 1 ($C_{-164}A$, *CYP1A2*1F*, Sachse *et al.*, 1999). The $G_{-3858}A$ was associated with a reduction in CYP1A2 activity in smokers,

while the C_{-164} variant, when present in the homozygous state, was associated with a higher inducibility in smokers. In transfection assays, the G_{-3591} substitution did not appear to lead to a change in *CYP1A2* promoter activity (Chapter 4; Aitchison *et al.*, 2000a).

In a preliminary study on the $T_{-3591}G$ SNP and response to clozapine, I had found a trend for an association between the –3591T allele and response to clozapine in Caucasians (Fisher exact test, 1-tailed $P = 0.10$, OR = 3.00, exact 95% confidence limits 0.63-18.77; Aitchison *et al.*, 2001b). However, owing to the low frequency of the –3591G variant in this sample (0.02), and the fact that my transfection studies did not indicate that this polymorphism was associated with a functional effect, a further study, investigating the association between the -164C variant and clozapine response was undertaken (by EM Tsapakis, under my supervision). A preliminary analysis of this study has been reported (Tsapakis *et al.*, 2002b).

3.4.2 Aim

The C_{-164} variant has a relatively high allele frequency in a normal population (0.32, Sachse *et al.*, 1999), and has been associated with functional variation in CYP1A2 activity (altered inducibility). The aim of this study was to further investigate the association between polymorphism in *CYP1A2* and response to clozapine.

3.4.3 Methods

Two hundred and forty-eight patients treated with clozapine for at least 3 months were rated for global level of response to the medication by their prescribing consultants, categorising response into 1 of 4 categories: improved a lot (Category 1), improved a

little (Category 2), showed no improvement (Category 3), and inadequate information available (Category 4). In addition, the following clinical information was available for the sample: age, sex, ethnicity, age of onset of psychosis before 19 years, family history of schizophrenia in a first degree relative, and reason for commencement of clozapine. Venous blood samples were collected from each patient and genomic DNA extracted by standard procedures. Genotyping for the $C_{-164}A$ substitution was by PCR-RFLP assay, as described (Sachse *et al.* 1999). In brief, primers P1f (5'-CAACCCTGCCAATCTCAAGCAC, located in exon 1) and P4r (5'-AGAAGCTCTGTGGCCGAGAAGG, located in exon 2) were used to amplify up a 370 bp region of intron 1 of *CYP1A2*, PCR products then being digested with *Bsp*120I (MBI Fermentas). The reaction conditions and cycling parameters were as described by Sachse *et al.* (1999).

The statistical analyses were performed using SPSS (version 8.0) and EpiInfo (version 6.0). Data were analysed by genotype using a recessive model (C/C versus A/C or A/A) and by allele, and by logistic regression controlling for confounding variables. The recessive model was chosen as the C allele was associated with higher inducibility when present in the homozygous state in the study of Sachse *et al.* (1999). The genotypic distribution was tested for deviation from Hardy-Weinberg equilibrium using the χ^2 test (using the HWE programme), and a power calculation was performed using Solo Power Analysis.

3.4.4 Results

Of the 248 patients, 220 were Caucasian (85.3%), 12 African or Caribbean in origin, 10 Asian, and 6 of mixed race. One hundred and sixty-seven (67.3%) were male, 81

female (32.7%). The mean age of the whole sample was 38.7 years (sd 10.1). One hundred and forty-seven (59.3%) were in Category 1 in terms of response, 86 in Category 2 (34.7%), and 15 in Category 3 (6%); there were none in Category 4 (all subjects that had been on clozapine for at least 3 months had adequate response information). For the purpose of the analysis, subjects in Category 1 were deemed to be "responders," and subjects in Category 2 and 3 "non-responders." Thirty-six (14.5%) had a family history of schizophrenia in a first degree relative, 163 did not (65.7%), and for 49 (19.8%), this information was not available. Two hundred and twenty-four (90.3%) were commenced on clozapine due to being refractory to treatment with previous antipsychotics, 11 (4.4%) due to intolerance of previous antipsychotics, and 12 (4.8%) due to being both treatment-refractory and treatment-intolerant, and for 1 (0.4%) subject this information was not available. One hundred and nine subjects (43.5%) had their onset of psychosis before the age of 19 years (43.5%), 85 (34.3%) had onset later than 19 years, and for 55 subjects (22.2%) this information was not available.

The allele frequencies of the C and A allele in the whole sample were 0.31 and 0.69, in the Caucasians were 0.31 and 0.67, in the subjects of Afro-Caribbean origin 0.25 and 0.75, and in the Asians 0.35 and 0.65. The allele frequencies in the Caucasians (with schizophrenia or schizoaffective disorder) were very similar to those previously reported by Sachse *et al.*(1999) in normal subjects (C = 0.32; A = 0.68, N = 236) and to those reported by Basile *et al.* (2000) in Caucasians with schizophrenia (C = 0.34; A = 0.66, N = 63). Although there was a tendency for the Afro-Caribbeans (N = 12) to have a lower frequency of the C allele (which is in the opposite direction to that in the sample of 22 African-Americans reported by Basile *et al.* (2000), owing to the small

sample sizes in both of these studies, this was not deemed to be of any significance. There was no significant difference between the allelic distributions in the three ethnic groups ($\chi^2 = 0.56$, 2 degrees of freedom, $P = 0.76$). The genotypic distribution did not differ from that expected by Hardy-Weinberg equilibrium for the whole sample ($\chi^2 = 0.28$, 1 degree of freedom, $P = 0.30$), the Caucasian subset ($\chi 2 = 1.33$, $P = 0.12$), and the Afro-Caribbeans ($\chi^2 = 1.33$, $P = 0.12$). For the Asians, the distribution was just significantly different from Hardy-Weinberg equilibrium ($\chi^2 = 2.90$, $P = 0.04$), which may be artefactual due to the very small sample size (N = 10). The sample was analysed as a whole, with analysis for the Caucasians (the major ethnic subset) also being presented separately.

The results by genotype and by allele are shown in Tables 3.16 and 3.17. Grouping together responder categories 2 and 3 ("non-responders"), and analysing these versus category 1 ("responders") by genotype, using a recessive model, genotype frequencies did not differ significantly between responders and non-responders to clozapine treatment for either the whole sample ($P = 0.55$), or the Caucasians ($P = 0.70$). Analysis by allele also gave non-significant results, $P = 0.57$ for the whole sample, and $P = 0.90$ for the Caucasians.

The results of the logistic regression analysis versus dichotomised response as the dependent variable (Category 1 being "responders," Categories 2 and 3 being "non-responders"), controlling for the potential confounding factors of age, gender, ethnicity,

Table 3.16 *CYP1A2* $C_{-164}A$ results by genotype, in the three categories of response to clozapine in the sample, results presented for the whole sample and for the Caucasians (numbers in each cell with percentages given in parentheses)

	All ethnicities (N = 248)[1]			**Caucasians (N = 220)[2]**		
Response category	**A/A**	**A/C**	**C/C**	**A/A**	**A/C**	**C/C**
Improved a lot (Category 1)	72 (49.0)	61 (41.4)	14 (9.5)	64 (48.8)	53 (40.4)	14 (10.7)
Improved a little (Category 2)	39 (45.3)	36 (41.9)	11 (12.8)	36 (48.6)	28 (37.8)	10 (13.5)
No improvement (Category 3)	8 (53.3)	6 (40.0)	1 (6.7)	8 (53.3)	6 (40.0)	1 (6.7)
Percentage responders	*60.5*	*59.2*	*53.8*	*59.3*	*60.9*	*56.0*

Analysis conducted under recessive model for genotype, comparing Category 1 ("responders") versus Categories 2 and 3 ("non-responders"); [1]for whole sample, $\chi^2 = 0.35$, $P = 0.55$, odds ratio with 95% confidence limits = 1.28 (0.51 – 3.14); [2]for Caucasians, $\chi 2 = 015$, $P = 0.70$, odds ratio = 1.18 (0.46 – 2.96).

Table 3.17 *CYP1A2* $C._{-164}A$ results by allele, in the three categories of response to clozapine (allelic frequencies given in parentheses)

	All ethnicities (N = 496 alleles)[1]		Caucasians (N = 440 alleles)[2]	
Response category	**-164C**	**-164A**	**-164C**	**-164A**
Improved a lot (Category 1)	89 (0.30)	205 (0.70)	81 (0.31)	181 (0.69)
Improved a little (Category 2)	58 (0.34)	114 (0.66)	48 (0.32)	100 (0.68)
No improvement (Category 3)	8 (0.27)	22 (0.73)	8 (0.27)	22 (0.73)

Analysis by allele conducted comparing Category 1 ("responders") versus Categories 2 and 3 ("non-responders"), [1]for whole sample, $\chi^2 = 0.32$, $P = 0.57$, odds ratio = 1.12 (0.75 – 1.67); [2]for Caucasians, $\chi^2 = 0.01$, $P = 0.90$, odds ratio = 1.03 (0.66 – 1.58).

early onset psychosis (less than 19 years), family history of schizophrenia in a first degree relative, and reason why the patient was treated with clozapine, are given in Table 3.18. This revealed no significant association between response to clozapine and $C_{-164}A$ genotype. The only factor that emerged as significant in the logistic regression analysis was gender ($P = 0.03$, odds ratio = 0.52). Logistic regression analysis on the Caucasian subset also revealed a significant effect of gender only ($P = 0.03$, odds ratio = 0.50), and no effect of $C_{-164}A$ genotype ($P = 0.94$). The mean age did not differ significantly between subjects grouped by genotype (C/C: mean age 39.3, sd 10.08; C/A: mean age 38.7, sd 10.28; A/A: mean age 38.7, sd 9.98).

Table 3.19 shows the gender distribution in the different response categories; fifty-four per cent of males and sixty-nine per cent of women were responders. Chi square analysis (on a two by two table by combining response Categories 2 and 3) gave a significant effect of gender on response ($P = 0.03$, and OR = 0.53, exact 95% confidence limits 0.27-0.97), values that correlate well with the values derived from the logistic regression analysis (Table 3.18). This means that males are more likely to respond than females. Table 3.20 shows the –164C/A genotypic distribution in males and females, which shows that there is no significant difference between the two distributions ($P = 0.53$). This means that the effect of gender on response is not mediated by –164C/A genotype, which, again, is as expected from the logistic regression analysis.

3.4.5 Discussion

In a preliminary study, a trend towards an excess of the CYP1A2 G_{-3591} allele in those with a poor response to clozapine was found (Aitchison *et al.*, 2001b). This is a non-

Table 3.18 Results of logistic regression analysis, dependent variable dichotomised response ("responders," i.e. Category 1, versus "non-responders," i.e. Categories 2 and 3)

	Regression statistic			
Variable	**Regression coefficient**	**Wald score**	***P* value**	**Odds ratio**
CYP1A2 –164C/A genotype	0.11	0.28	0.59	1.11
Age	0.02	1.8	0.19	1.01
Gender	-0.65	4.93	0.03	0.52
Ethnicity	-0.01	0.004	0.95	0.98
Early onset psychosis (<19 years)	0.06	0.12	0.73	1.06
Family history of schizophrenia	-0.21	0.83	0.36	0.81
Reason for commencement of clozapine (i.e. intolerant or refractory to previous treatment)	-0.26	0.88	0.35	0.77

Table 3.19 Gender distribution in the different response categories (numbers with response rate for given gender per cell expressed as percentage)

	Gender	
Response category	**Male (N = 167)**	**Female (N = 81)**
Improved a lot (Category 1)	91 (54.5)	56 (69.1)
Improved a little (Category 2)	65 (38.9)	21 (25.9)
No improvement (Category 3)	11 (6.6)	4 (4.9)

Comparison of responders (Category 1) versus non-responders (Categories 2 and 3) gives $\chi^2 = 4.85$, $P = 0.028$, and OR = 0.53 (exact 95% confidence limits 0.29-0.97).

Table 3.20 Gender distribution by genotype (numbers plus percentage of given gender per cell)

	Gender	
CYP1A2 –164C/A genotype	**Male (N = 167)**	**Female (N = 81)**
A/A	76 (45.5)	53 (53.0)
A/C	73 (43.7)	30 (37.0)
C/C	18 (10.8)	8 (9.9)

Chi square analysis of gender distribution gives: $\chi^2 = 1.28$, 2 degrees of freedom, $P = 0.53$.

functional polymorphism in the *CYP1A2* 5' flanking region (Chapter 4). However, genotyping of the $C_{-164}A$ SNP in the *CYP1A2* intron 1 which has been associated with a high inducibility phenotype (Sachse *et al.*, 1999), did not indicate any association with clozapine response. Logistic regression, controlling for potential confounding factors, also revealed no significant association between response to clozapine and $C_{-164}A$ genotype ($P = 0.59$).

The possibility of a Type II error (false negative) has to be considered. The power calculation for χ^2, $df = 1$, shows that a sample size of 248, at the $P = 0.05$ level has a power of 0.997 to detect a medium effect size ($w = 0.30$), or 0.88 to detect a slightly smaller effect size ($w = 0.20$). The corresponding values for the Caucasian subset (N = 220) are 0.99 and 0.84. The power of the logistic regression analysis on the whole sample (N = 248, proportion of responders = 0.59, odds ratio set at 1.4, correlation 0.1, $P = 0.05$) is 0.84, and for the Caucasian subset 0.80. However, no data on cigarette smoking for this sample was available, and it may be that it is essential to have data on this to investigate a putative association with the C_{-164} SNP. Furthermore, it is possible that clozapine itself may induce CYP1A2, and therefore that information on the dose of clozapine (again, unavailable for this sample) and controlling for this variable in the analysis is required. I suggest that any future studies investigating putative associations between *CYP1A2* polymorphisms and response to clozapine should include collection of these clinical variables.

It is of note that the only factor that emerged as significantly associated with response was gender. The association of female gender with better response found in this study is contrary to the findings of Lieberman *et al.* (1994) on 84 patients (67% male);

however, in the latter study, the women had a longer duration of illness prior to clozapine treatment, which may independently be associated with relatively poor response. In another study on 49 men and 12 women, there was no difference in the gender distribution between responders and non-responders (Honer *et al.*, 1995). A poorer response in males could be due to factors such as the higher incidence of neurodevelopmental abnormalities in males (Castle & Murray, 1991; Murray, 1994), or, for example, the higher CYP1A2 activity is in males as compared to females (Sections 1.5.3.1 and 1.5.3.2). Consistent with this women were reported to have higher plasma concentrations of clozapine than men by Haring and colleagues (1989, 1990), and in a more detailed later analysis of therapeutic monitoring data on 162 Chinese patients with schizophrenia (Lane *et al.*, 1999), females had 35% higher clozapine levels and 36% higher norclozapine levels than males, with no sex differences being seen for clozapine *N*-oxide levels, and Jerling *et al.* (1997) also in a therapeutic drug monitoring analysis (391 samples from 241 patients) demonstrated higher clearance and larger volume of distribution in males as compared to females. The lower clearance and higher clozapine and norclozapine levels in women would be consistent with the lower CYP1A2 activity (see Chapter 5). This may at least partially account for the higher response rate in women, especially as there is a documented association between clozapine levels (of at least 350-420 μg/l) and response (Perry *et al.*, 1991; Hasegawa *et al.*, 1993; Kronig *et al.*, 1995; Miller *et al.*1994; Potkin *et al.*, 1994).

If there were a polymorphism in *CYP1A2* (or its flanking regions) related to the differential CYP1A2 activity between males and females, then it would be expected that response to clozapine would be associated with such a polymorphism. The mechanism of the effect of oestrogens on CYP1A2 is not well understood, but by

analogy with the effects of other steroids on regulation of gene expression, may be exerted by a transcriptional effect on the *CYP1A2* promoter. It is then possible that a *CYP1A2* promoter variant which is associated with the inhibition of CYP1A2 by a variety of agents including oestrogens might be associated with clozapine response. Haring *et al.* (1990) reported that smoking was associated with lower clozapine levels in men only, which might indicate that the mechanism of CYP1A regulation by smoking (involving the XRE sites) might overlap with the mechanism of CYP1A2 regulation by oestrogens. The $T_{-3591}G$ SNP might be in linkage disequilibrium with promoter variant(s) involved in such regulation. The trinucleotide repeat region, $(AAC)_6$ at –4382 to –4399 (section 4.5.2, and Figure 4.11), warrants investigation as a candidate for functional variation, and further mutation screening of the *CYP1A2* promoter, including the multiple putative XRE sites described by Corchero *et al.* (2001) and regions that are conserved across species and could be involved in the transcriptional effect of oestrogens, could yield interesting data.

CHAPTER FOUR

CYP1A2 NOVEL MUTATION SCREENING AND FUNCTIONAL CHARACTERISATION

4.1 Introduction

4.1.1 Interindividual variation in CYP1A2

There is wide interindividual variation in CYP1A2 activity (Kalow and Tang, 1991a), which in most studies has been demonstrated to be trimodally or bimodally distributed (Butler *et al.*, 1992; Lang *et al.*, 1994; Nakajima *et al.*, 1994; Schrenk *et al.*, 1998; Ou-Yang *et al.*, 2000). Butler and colleagues (1992) studied individuals from Arkansas, Italy, and China, and found that CYP1A2 activity was trimodally distributed, with the range of percentages in the 3 populations being: 12-13% slow, 51-67% intermediate, and 20-37% rapid metabolisers. Nakajima *et al.*, 1994 studied 205 Japanese and found a bimodal distribution of CYP1A2 activity in both smokers and non-smokers, with 13.8% of smokers and 14.3% of non-smokers being poor metabolisers (PMs), the rest being termed extensive metabolisers (EMs). An earlier UK study had found that 10% of the population was deficient in phenacetin *O*-deethylase activity (Devonshire *et al.*, 1983), which had been subsequently shown to be CYP1A2 dependent (Distlerath *et al.*, 1985). Studies in monozygotic and dizygotic twins indicated a high heritability (h_1 = 0.83) for an index of CYP1A2 activity (urinary excretion of 3-methylxanthine; Miller *et al.*, 1984). A genetic polymorphism to account for this variability was suggested by an early family study in which impaired phenacetin-*O*-deethylation was noted in 2 siblings (Shahidi, 1968), and Nakajima and colleagues (1994) extended this work to

study the pattern of CYP1A2 activity in 8 pedigrees, the results of which were consistent with genetic polymorphism at a single gene locus, with autosomal codominant transmission.

At least some of the interindividual variability in CYP1A2 activity is, however, explicable by environmental factors. The enzyme may be inhibited or induced by various dietary substances, drugs, and toxins (section 1.5.3.1). Nonetheless, Le Marchand and colleagues (1997) showed in a study of 90 subjects of various ethnic backgrounds in Hawaii that 73% of the variance remained unexplained after taking into account the major environmental contributors to the variance. In a larger study (N=786 Caucasians), Tantcheva-Poór *et al.* (1999) found that 63% of the overall variation remained unaccounted for after analysis for the major covariates (*e.g.* coffee consumption). This finding points to the existence of other factors, such as genetic polymorphisms, as contributors to the variation in indices of CYP1A2 activity.

4.1.2 Interethnic variation in CYP1A2

There is evidence for interethnic variation in CYP1A2 activity. Butler and colleagues (1992) found that the frequency of rapid metabolisers in non-smokers was 20% in 50 Italians, and 37% in 77 individuals from Arkansas; Le Marchand *et al.* (1997) in their study found a significantly higher mean CYP1A2 activity in 15 Caucasians as compared with 45 Japanese; Relling *et al.* (1992) showed that CYP1A2 activity was significantly lower in a group of 63 Black subjects in comparison to a group of 246 White subjects, and Lang *et al.* (1994) found African-American smokers had CYP1A2 activity approximating that of African American or Caucasian non-smokers. Furthermore, a study of clozapine levels in 162 Taiwanese found mean plasma

concentrations 30-50% higher than those reported in Caucasians for equivalent doses (Chang *et al.*, 1997).

4.1.3 Previous mutation screening of *CYP1A2*

In their 1994 paper, Nakajima and colleagues, using PCR followed by direct sequencing, screened the exons, exon-intron junctions, and the 5'flanking region to –3470 bp in 2 non-smoking PMs, 1 non-smoking EM, 1 smoking PM, and 1 smoking EM and found no differences in the nucleotide sequence between each phenotype. Subsequent studies (see section 4.6) had not yet been performed by the time I performed this part of the work for my thesis.

4.1.4 Aims of this study

The majority of the data in this study has already been reported (Aitchison *et al.*, 2000a). Given the indication that factors including genetic polymorphism in *CYP1A2* could contribute to CYP1A2 variability and the negative finding of Nakajima *et al.* (1994), I decided to screen the 5' flanking region of *CYP1A2* further upstream than –3470 bp[1]. TCDD induces the expression of *CYP1A1* as well as that of *CYP1A2*, and in this case, has been shown to exert this effect via its interaction with xenobiotic responsive elements (XREs) in the promoter sequence. Two XRE-like sequences have been identified in the human *CYP1A2* promoter, at positions –3382 to –3400, and –3037 to –3055 (Quattrochi *et al.*, 1994). These sequences were shown to lie within regions that contribute to the induction of *CYP1A2* by 3-methylcholanthrene (Quattrochi *et al.*, 1994), and in transient transfections, TCDD was found to induce the *CYP1A2* promoter (Postlind *et al.*, 1993). However, the fold induction is much higher for *CYP1A1* than for *CYP1A2* (using a *Kpn*I fragment of *CYP1A2*, -4096/-842), and

induction of *CYP1A2* was not seen in stable transfectants (Postlind *et al.*, 1993). I therefore decided to determine the sequence of the *CYP1A2* 5' flanking region that was further upstream than –4096, to see whether or not another XRE, polymorphism in which could affect the inducibility of CYP1A2, could be found.

[1]I have numbered the nucleotides in the *CYP1A2* 5' flanking region counting the start of translation as +1 (position 2804 in GenBank sequence accession number M31664), in accordance with the international nomenclature for human *CYP1A2* alleles, available at www.imm.ki.se/CYPalleles.

4.2 Mutation screening of the *CYP1A2* 5' flanking region

4.2.1 Study design

Three sample groups were used for this study: 87 Caucasians from the UK (section 2.1.1.2), 125 Taiwanese (section 2.1.1.3), and 105 African Americans (section 2.1.1.4). In addition, a few Caucasians (staff of the laboratory of Dr FJ Gonzalez) were used in the initial mutation screening, but not included in the analysis of the frequency of the $T_{-3591}G$ SNP in different ethnic groups. The method of mutation screening was PCR-based resequencing, with an initial 11 Caucuasians and 8 Taiwanese being screened (section 4.2.3), and a total of 20 Caucasians, 31 Taiwanese, and 10 African Americans being sequenced in all (section 4.2.4). Sequence further upstream than the 5' flanking sequence of CYP1A2 that was publicly available at the time that I commenced my work was obtained initially by Genome Walking (section 4.2.2), which was conducted at the Institute of Psychiatry (UK), and latterly by direct sequencing of BAC clones (section 4.5), conducted at the laboratory of Dr FJ Gonzalez (NIH, USA).

4.2.2 Genome walking

The methodology for genome walking is given in section 2.2.6. Sequence from the second PCR product of library 2 produced sequence which aligned with the 5' flanking sequence of *CYP1A2*, and revealed the following sequence discrepancies compared with the published sequence: a $G_{-3649}C$ substitution and a T_{-3650} deletion.

4.2.3 PCR sequencing

I investigated these potential polymorphisms ($G_{-3649}C$ and ΔT_{-3650}) by PCR sequencing (section 2.2.7) using PCR reaction M2-M1 with sequencing primers M1 and M2 (Table 4.1 and Figure 4.1), and found the same sequence discrepancies in 11 Caucasians and 8 Taiwanese samples. Furthermore, these discrepancies were found in plasmid pL1A2N supplied by Dr Linda Quattrochi, which is a derivative of plasmid pH4CAT1, the latter being the plasmid that was sequenced by Quattrochi and Tukey (1989) in their original publication of the *CYP1A2* 5' flanking sequence, and hence these sequence discrepancies are likely to represent errors in the original published sequence.

However, the above PCR sequencing also revealed a $T_{-3591}G$ substitution in one of the Caucasians and one of the Taiwanese. This Caucasian was one of the Gonzalez laboratory staff members, and was homozygous for the $T_{-3591}G$ substitution (Figure 4.2 shows the wild-type reverse sequence in this region, Figure 4.3 the reverse sequence of this individual). Further PCR sequencing using PCR reactions M3-M4 or cyp1abr2-M5 with sequencing primers M1, M3, or M5 also revealed a second polymorphic site in the5' flanking region of *CYP1A2*, a $G_{-3595}T$ substitution, in one out of a further 20 Caucasian subjects, three out of a further 31 Taiwanese subjects, and three out of 10 African American subjects sequenced. The Caucasian and the three Taiwanese subjects

Table 4.1 Primers and annealing conditions used for genome-walking from, polymerase chain reaction amplification of, and site-directed mutagenesis of the *CYP1A2* 5' flanking region. Underlined nucleotides are the mutant sites in the primers used for site-directed mutagenesis.

Location of Primers		**Sequence**	**Annealing conditions**
Cyp1abr2	-3387 → -3411	5'-GTGCGTGTCAGGTCTCTTCACTGTA-3'	72 ^{0}C, 3 min and 67 ^{0}C, 3 min (see GenomeWalker kit manual)
Cyp1abr1	-3292 → -3316	5'-GGTTAGGTGCCATTCTCGTCACATC-3'	
M2	-3798 → -3820	5'-GCTGGAATTACAGGTGTGCACCA-3'	55 ^{0}C, 1 min
M1	-3545 → -3562	5'-CTCCTGTATGACAGACTA-3'	
Cyp1abf	-3880 → -3899	5'-GTGCAGTGGTGCGATCTTGG-3'	54 ^{0}C, 30 s (with cyp1abr2)
M3	-3454 → -3472	5'-GGAAGTCACTATGCACAGC-3'	55 ^{0}C, 1 min (with M2)
M4	-3706 → -3726	5'-GAACTCCTGGCCTCACTCAAG-3'	53 ^{0}C, 30 s (with M3)
M5	-3633 → -3651	5'-GTCCCAGCTGAC ATATGCA-3'	53 ^{0}C, 30 s (with cyp1abr2)
SDM1F	-3568 → -3616	5'-CCTGTAATTTAATTTTTTTAAGTTTGAAGAAAACATTAAAAATAAAAAG-3'	55 ^{0}C, 1 min
SDM1R	-3568 → -3616	5'-CTTTTTATTTTTAATGTTTTCTTCAAACTTAAAAAAATTAAATTACAGG-3'	
SDM2F	-3583 → -3627	5'-CTATATTGTATCCTGTAATTTAATTTTTTTTAATTTTTAAGAAAAC-3'	55 ^{0}C, 1 min
SDM2R	-3583 → -3627	5'-GTTTTCTTAAAAATTAAAAAAATTAAATTACAGGATACAATATAG-3'	
SDM3F	-3583 → -3627	5'-CTATATTGTATCCTGTAATTTAATTTTTTTTAATTTTGAAGAAAAC-3'	55 ^{0}C, 1 min
SDM3R	-3583 → -3627	5'-GTTTTCTTCAAAATTAAAAAAATTAAATTACAGGATACAATATAG-3'	

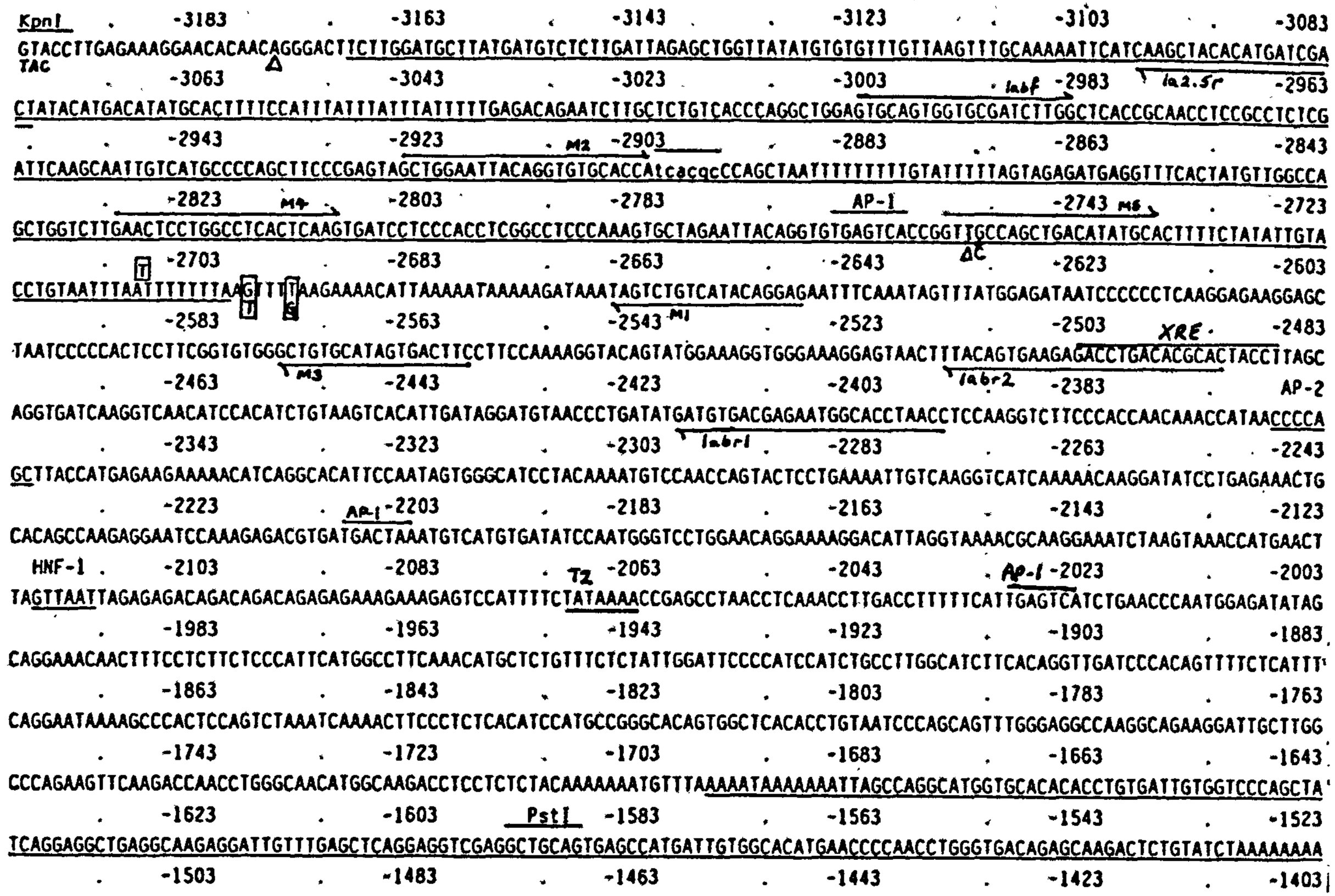


Figure 4.1 Sequence of the 5' flanking region of *CYP1A2*, from Quattrochi & Tukey (1989), with the positions of primers that I used marked, and also the sequence discrepancies and polymorphisms identified; XRE = xenobiotic responsive element; AP-1 = AP-1 binding site.

were heterozygous for both of the point mutations (giving an allele frequency of 0.025 for the $G_{-3595}T$ in the Caucasians, and 0.048 in the Taiwanese; Figure 4.4), while two of the African American subjects were homozygous for the $G_{-3595}T$ substitution (and wild type for the T_{-3591}; Figure 4.5), and one was heterozygous for the $G_{-3595}T$ substitution (and wild type T_{-3591}), which gives an allele frequency of 0.25 for the $G_{-3595}T$ in this African American subgroup. (The numbers of samples in the 3 populations for which I have data on both the $T_{-3591}G$ and $G_{-3595}T$ polymorphisms are too small to enable calculation of whether or not the 2 polymorphisms are in linkage disequilibrium.)

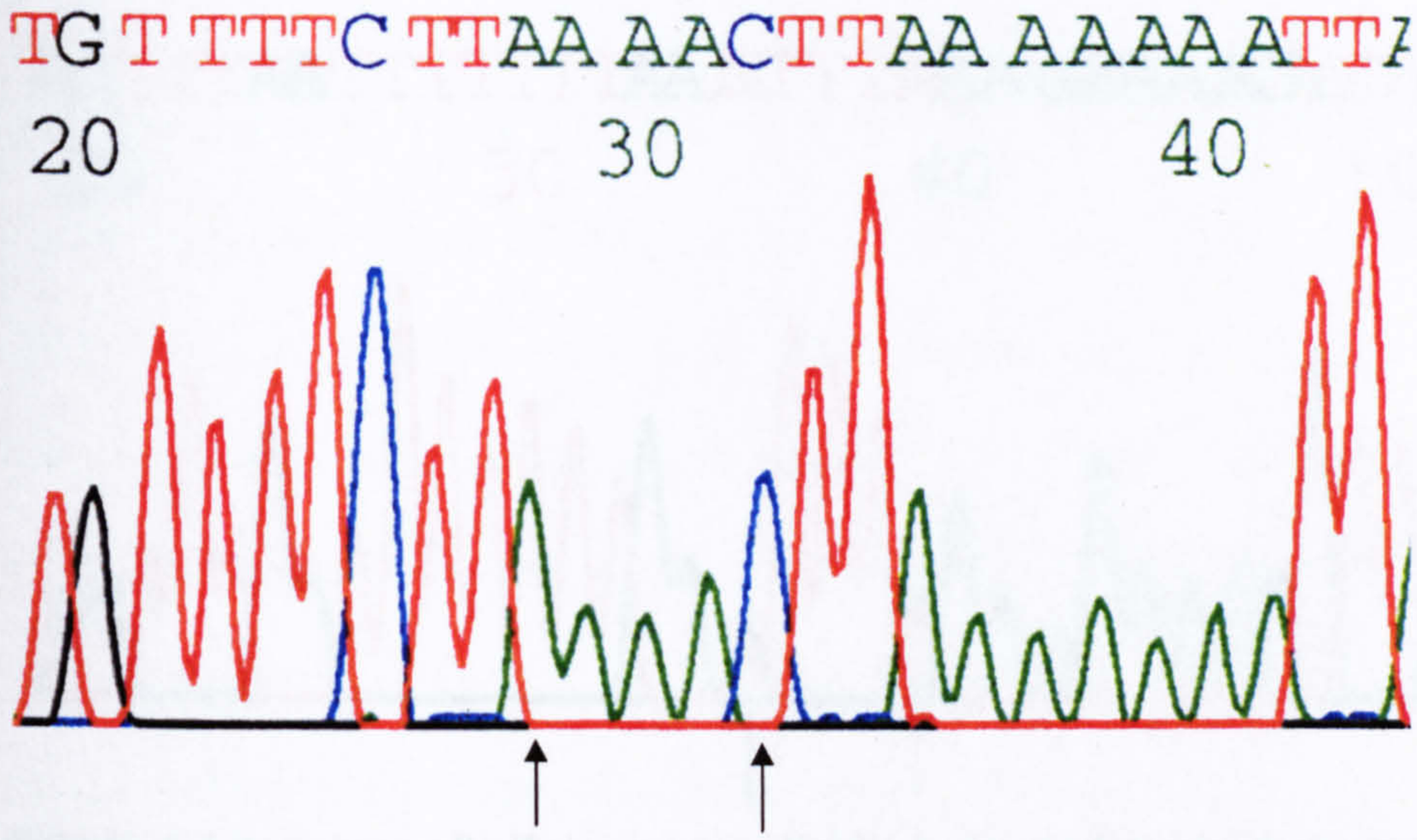


Figure 4.2 Sequence of 5'flanking region of *CYP1A2*, reverse strand, of an individual wild-type for both the $T_{-3591}G$ (*i.e.* A_{-3591} on reverse strand, sequence position 28) and $G_{-3595}T$ (*i.e.* C_{-3595}, sequence position 32) SNPs.

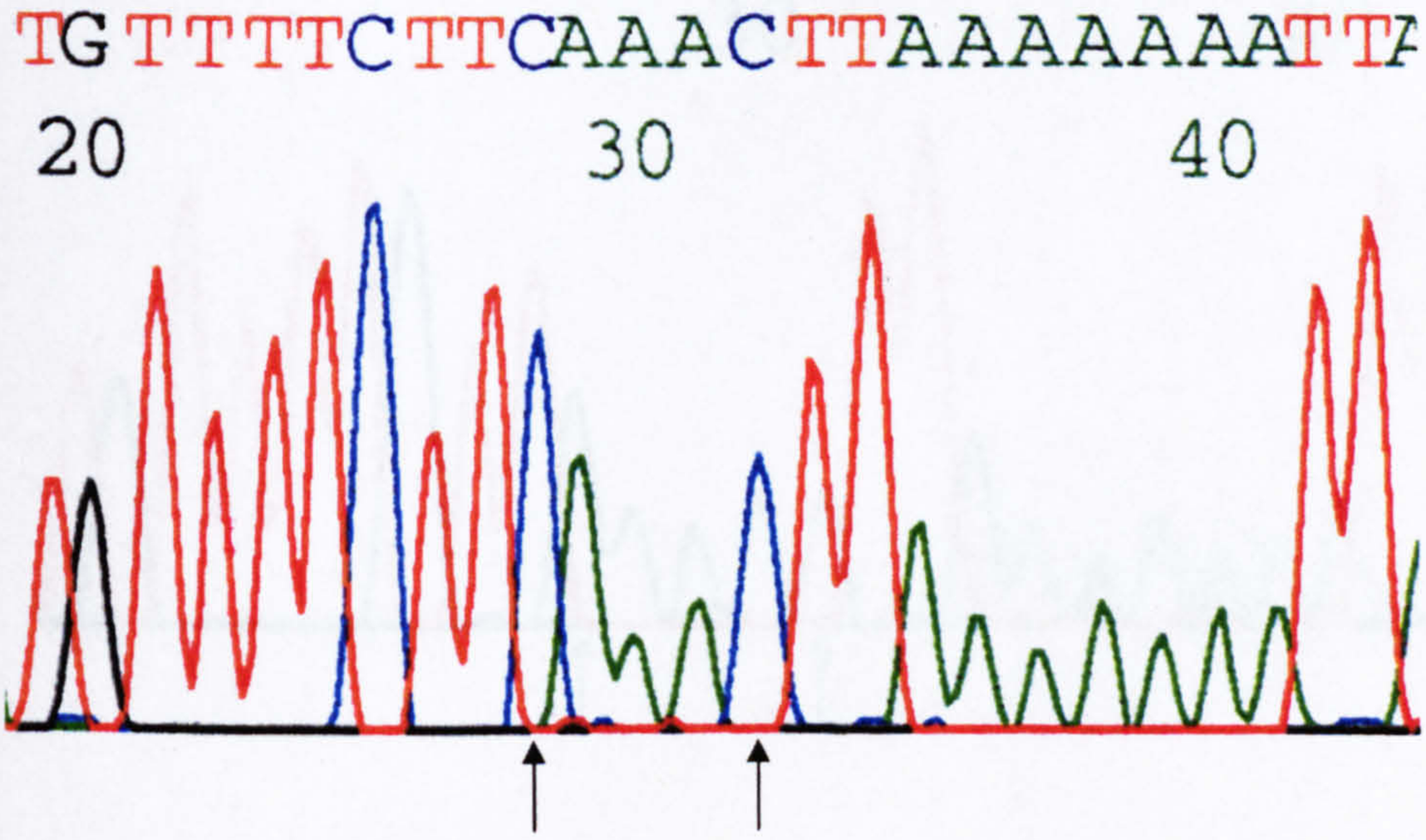


Figure 4.3 Sequence of 5'flanking region of *CYP1A2*, reverse strand, of the Caucasian individual homozygous for the $T_{-3591}G$ (*i.e.* C_{-3591} on reverse strand, sequence position 28) and wild-type for the $G_{-3595}T$ (*i.e.* C_{-3595}, sequence position 32) substitution

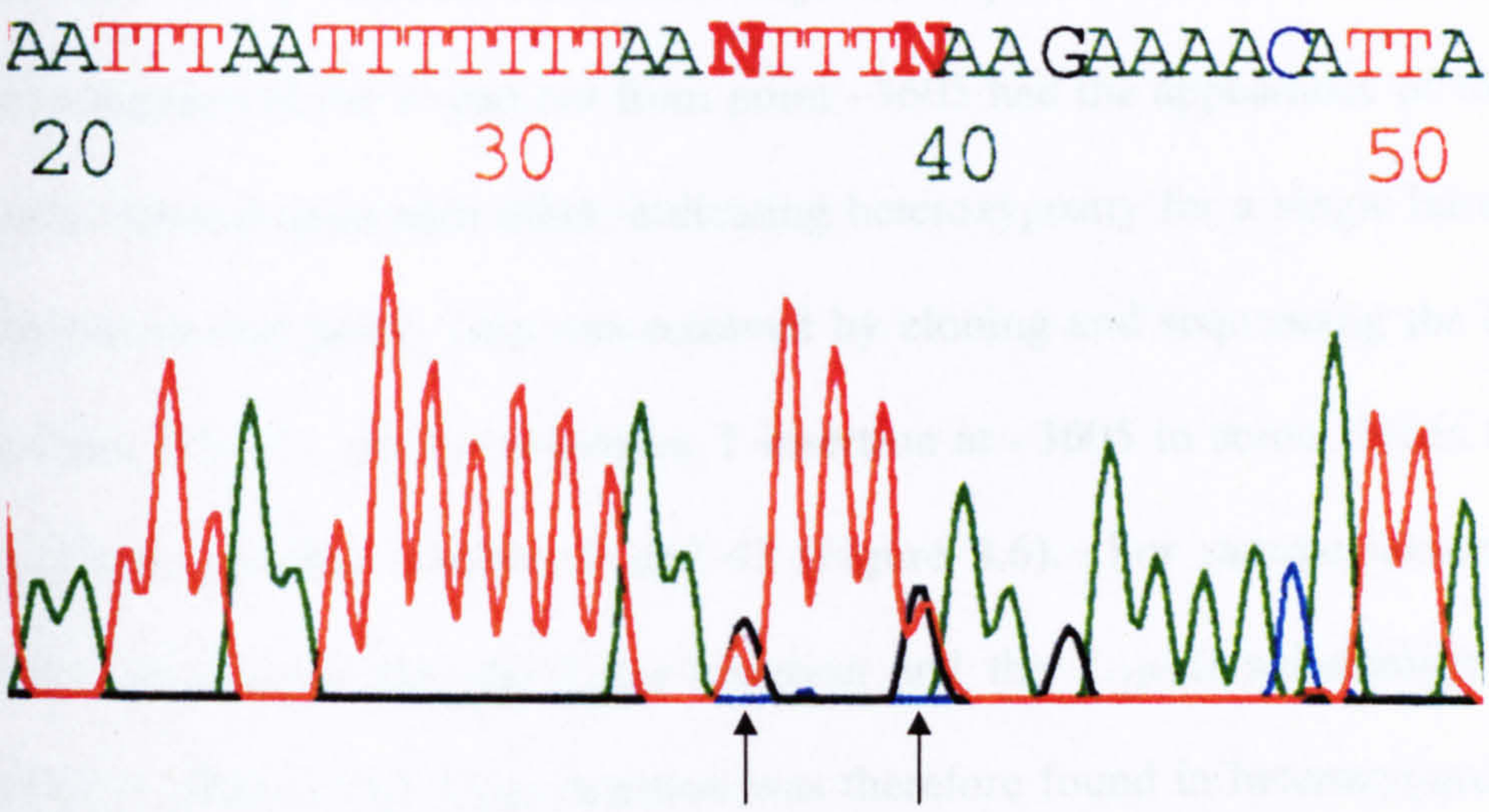


Figure 4.4 Sequence of a Taiwanese individual, forward strand, heterozygous for the T.$_{3591}$G and for the G.$_{3595}$T substitutions (sequence positions 35 and 39)

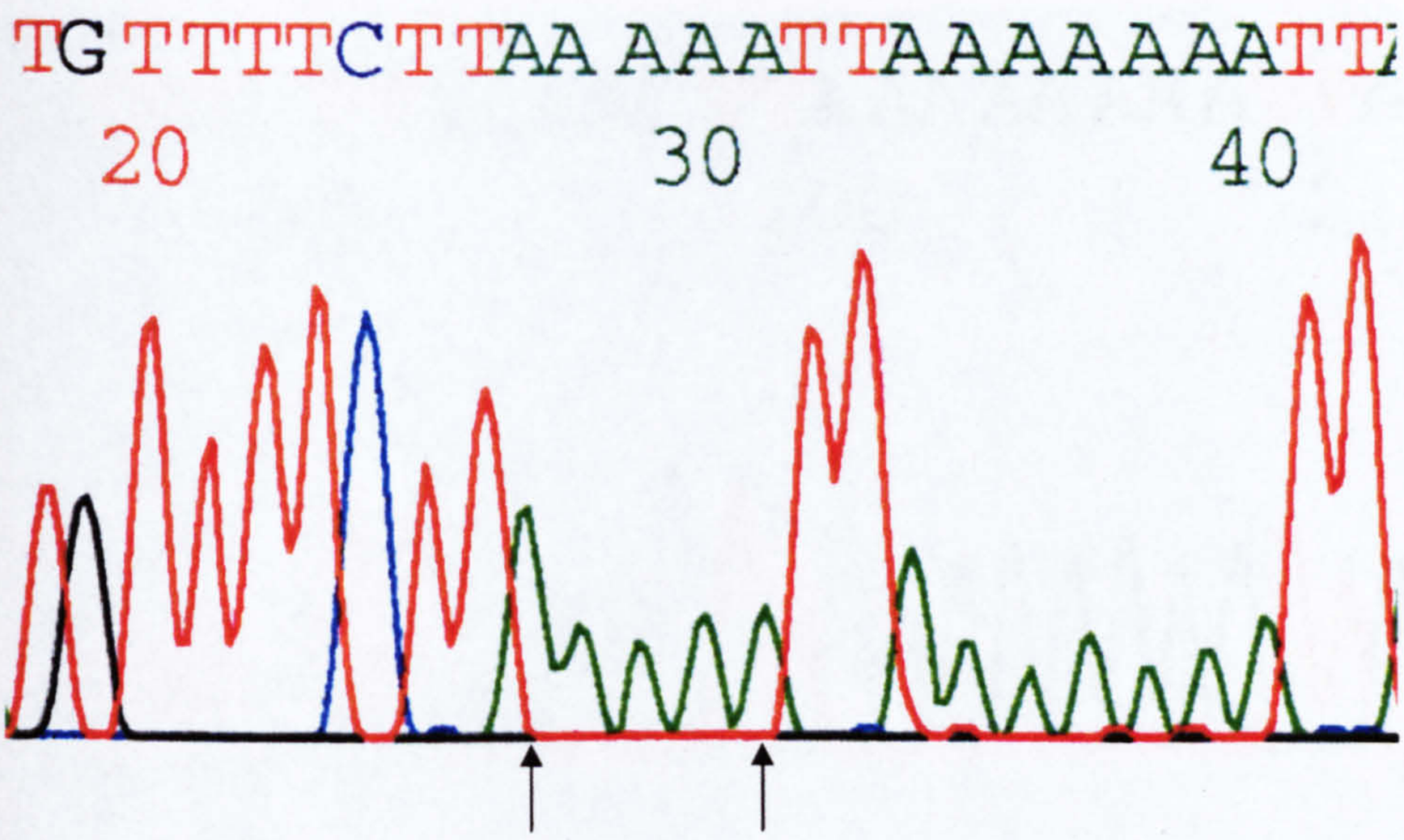


Figure 4.5 Sequence of 5'flanking region of *CYP1A2*, reverse strand, of an African American individual wild-type for the T.$_{3591}$G (*i.e.* A.$_{3591}$ on reverse strand, position 27 on sequence trace) and homozygous for the G.$_{3595}$T (*i.e.* A.$_{3595}$, position 31 on sequence trace) substitution

4.2.4 Further mutation identification and cloning

For two of the African American subjects, sequence in the reverse direction was unambiguous at the 3' end but from point –3605 had the appearance of two sequences superimposed upon each other, indicating heterozygosity for a single base insertion or deletion at that point. This was resolved by cloning and sequencing the PCR product (section 2.2.8.1), which revealed a T insertion at –3605 in some clones from African American samples numbers 9 and 43 (Figure 4.6). For sample number 43, *Mbo*II digestion showed that the T_{-3605} insertion and the $T_{-3591}G$ substitution occurred on different alleles. The T_{-3605} insertion was therefore found in heterozygous state in two out of 10 African Americans sequenced (giving an allele frequency of 0.10), in 0 out of 31 Taiwanese sequenced, and in 0 out of 20 Caucasians sequenced.

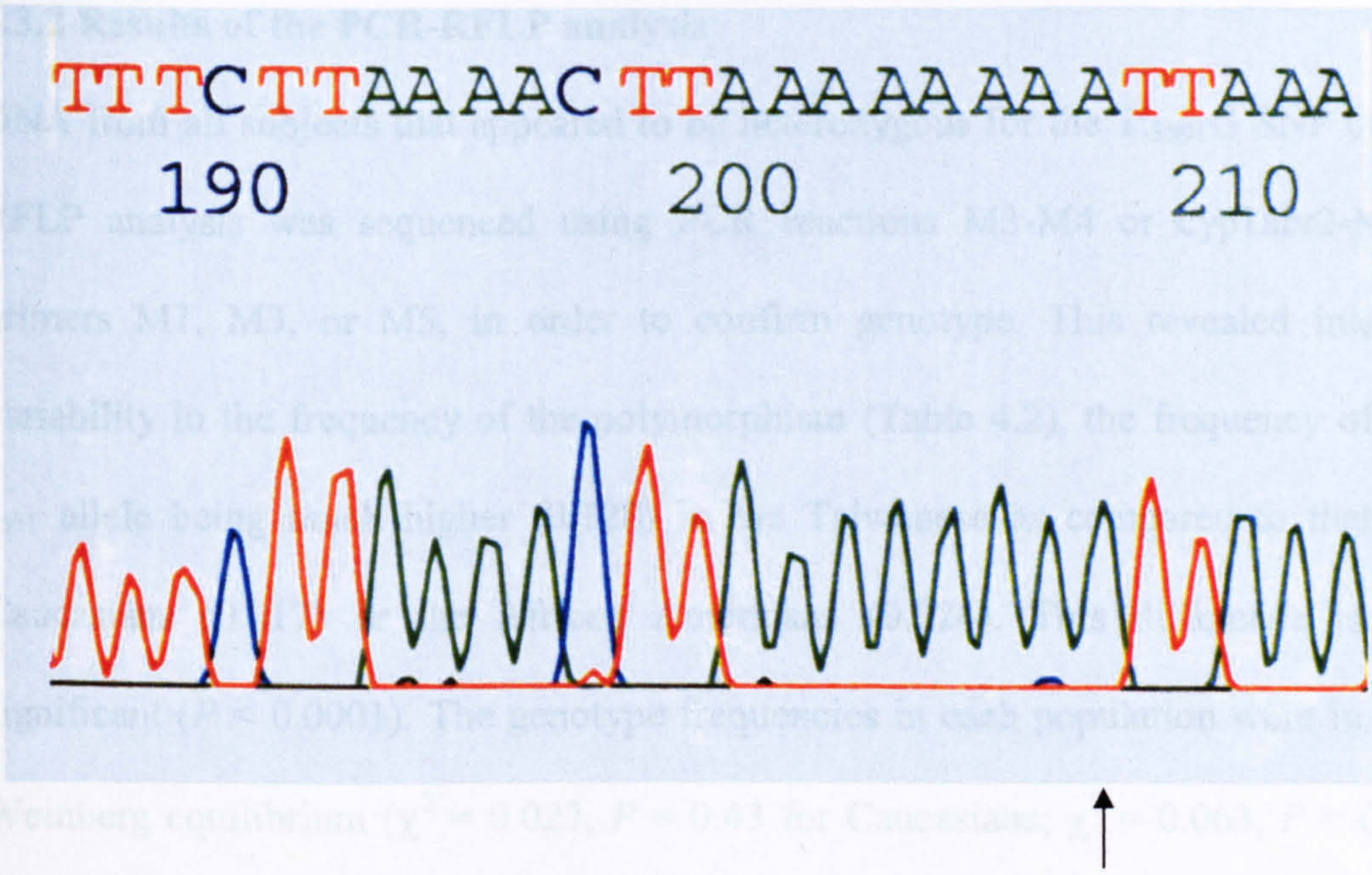


Figure 4.6 Sequence of one clone from M3-M4 PCR product of African American sample number 43 (reverse strand), showing an A_{-3605} insertion (*i.e.* T_{-3605} insertion on forward strand)

4.3 Determination of the frequency of the $T_{-3591}G$ mutation in different ethnic groups

4.3.1 Development of a PCR-RFLP assay

In order to investigate the frequency of the $T_{-3591}G$ SNP in the 3 sample groups, I designed a PCR-RFLP assay. This involved a nested PCR, the first PCR reaction using primers cyp1abr2 and cyp1abf, the second PCR reaction using primers M2 and M3 (Table 4.1, Figure 4.1). Products were digested with *Mbo*II (New England Biolabs) and separated on a 3% agarose gel (Figure 4.7; the G_{-3591} substitution creates an *Mbo*II site). I did not screen for the remaining 2 polymorphisms, as I felt this to be inappropriate before their functional significance was known.

4.3.2 Results of the PCR-RFLP analysis

DNA from all subjects that appeared to be heterozygous for the $T_{-3591}G$ SNP on PCR-RFLP analysis was sequenced using PCR reactions M3-M4 or cyp1abr2-M5 and primers M1, M3, or M5, in order to confirm genotype. This revealed interethnic variability in the frequency of the polymorphism (Table 4.2), the frequency of the G_{-3591} allele being much higher (0.128) in the Taiwanese as compared to that in the Caucasians (0.017) or the African Americans (0.024). This difference is highly significant ($P < 0.0001$). The genotype frequencies in each population were in Hardy-Weinberg equilibrium ($\chi^2 = 0.027$, $P = 0.43$ for Caucasians; $\chi^2 = 0.063$, $P = 0.40$ for African Americans; $\chi^2 = 2.45$, $P = 0.06$ for Taiwanese). For the genotypic comparisons, I grouped together the heterozygotes with the homozygous mutants because if the mutation was associated with reduced or increased promoter activity and hence reduced or increased enzyme activity, then, by analogy with findings of other variant alleles in

the cytochromes (*e.g. CYP2D6*, Suzuki *et al.*, 1997), the possession of a single mutant allele could be associated with a degree of variation in enzyme activity (*i.e.* treating the mutant allele as dominant). Furthermore, as there were no homozygous mutants (-3591G/G) in either the Caucasians or the Black Americans, statistical analyses comparing the frequency of homozygous mutants with the frequencies of the other genotypes in these 2 groups were meaningless.

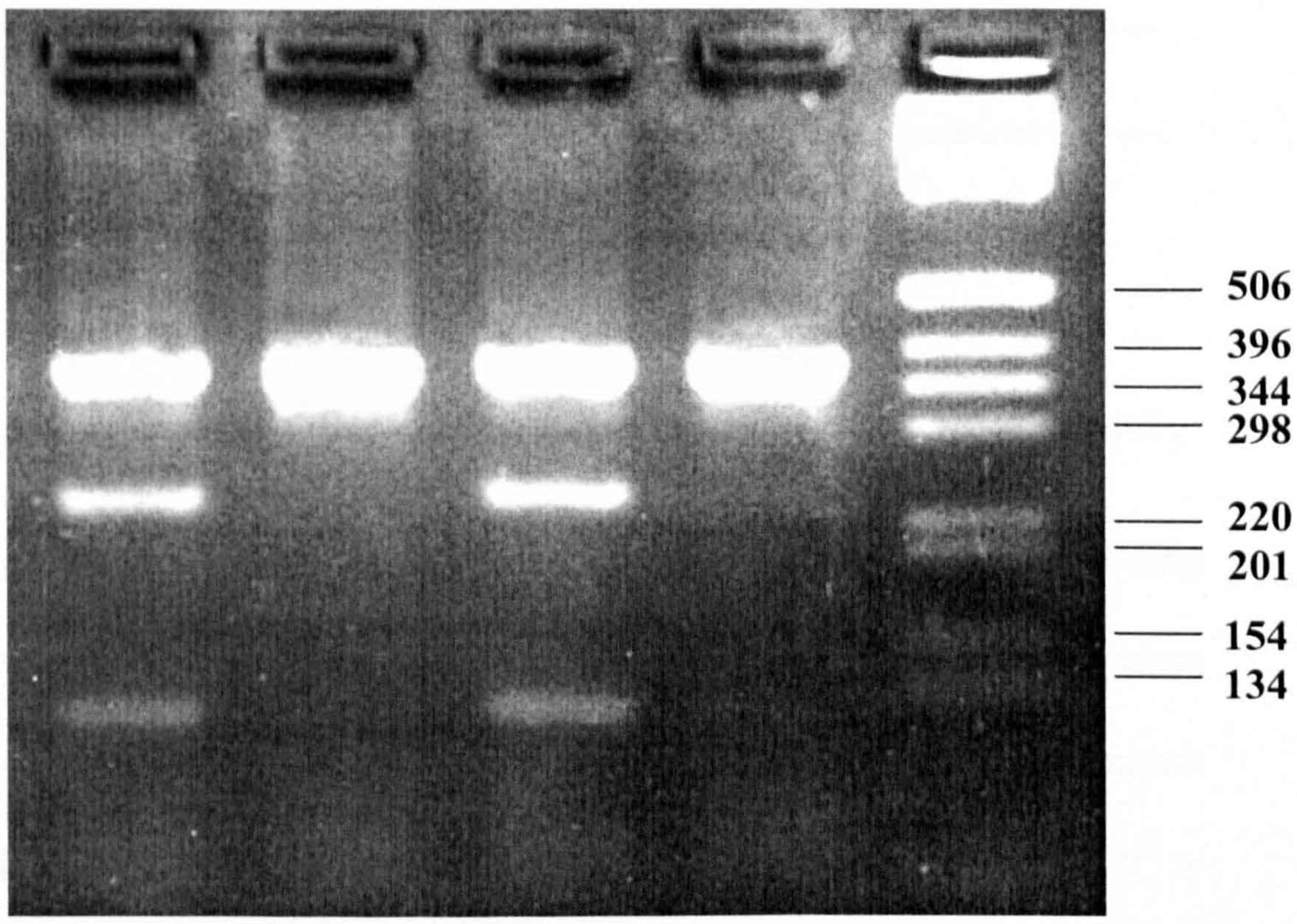


Figure 4.7 Agarose gel electrophoresis of *Mbo*II digestion of M2-M3 PCR products. Subjects in lanes 1 and 3 are heterozygous for the $T_{-3591}G$ substitution; subjects in lanes 2 and 4 are homozygous wild type; in lane 5 is a 1 kb ladder (Gibco BRL, bp).

Table 4.2 *CYP1A2* $T._{3591}G$ genotypes in 87 Caucasians, 104 African Americans, and 125 Taiwanese

	Homo *mut*	Het *mut*	Homo *wt*
Caucasian	0	3	84
African American	0	5	99
Taiwanese	4	24	97

Mut, mutant, $G._{3591}$ allele; *wt*, wild type, $T._{3591}$ allele. Analysis by genotype, comparing homozygous mutant or heterozygous mutant versus wild type, Caucasians versus Taiwanese: $\chi^2 = 14.8$, $P = 0.0001$. By allele, comparing Caucasians versus Taiwanese: $\chi^2 = 16.6$, $P < 0.0001$. For African Americans versus Taiwanese by genotype, $X^2 = 14.2$, $P = 0.0001$ and by allele $\chi^2 = 16.5$, $P < 0.0001$. For Caucasians versus African Americans, by genotype and by allele, Fisher's exact test gives a 1-tailed *P*-value of 0.46.

4.4 Investigation of the functional effect of the $T_{-3591}G$ and $G_{-3595}T$ substitutions

4.4.1 Site-directed mutagenesis

I decided to investigate the functional significance of the $T_{-3591}G$ and $G_{-3595}T$ mutations as these were the more frequent of the mutations identified. I performed site-directed mutagenesis (section 2.2.8.2) to create 3 mutant plasmids: SDM1 (with the $T_{-3591}G$ substitution), SDM2 (with the $G_{-3595}T$ substitution), and SDM3 (with both substitutions), the latter plasmid being created as sequencing had already identified samples in which both of these mutations were present, and hence the functional consequence of this was of interest.

4.4.2 Transient transfections

Transient transfections were conducted using HepG2 cells (section 2.2.9), setting up parallel experiments with wild-type plasmid (pL1A2N), SDM1, SDM2, and SDM3, and treating 50% of the cells with a CYP1A2 inducer (TCDD).

4.4.3 Results of transient transfections

The results of the transient transfection experiments using HepG2 cells showing the constitutive (non-induced) levels of luciferase (reporter product) activity and induced (TCDD-treated) levels are shown in Figure 4.8.

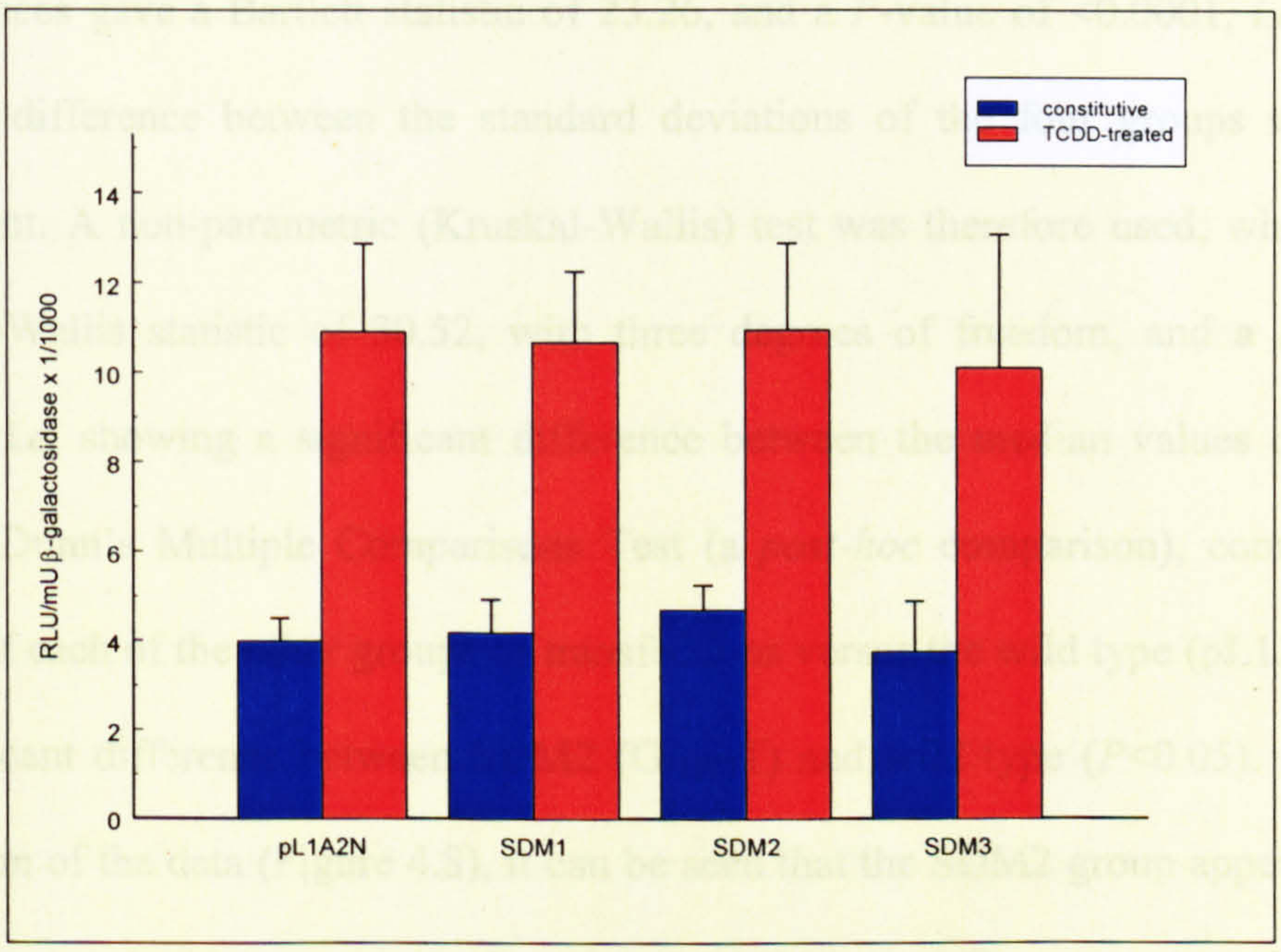


Figure 4.8 Results of transient transfections of HepG2 cells with luciferase reporter vectors containing the *CYP1A2* promoter; pL1A2N is the wild type promoter; SDM1, SDM2, and SDM3 were created by site-directed mutagenesis of pL1A2N. SDM1 contains the $T_{-3591}G$ substitution, SDM2 the $G_{-3595}T$ substitution, and SDM3 both substitutions. Cells were cotransfected with a CMVβ plasmid (expressing β-galactosidase); results are given as relative light units (RLU) per milliunit (mU) of β-galactosidase x 10^{-3}. The TCDD-treated groups were treated with 10 nM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin at 40 hours post transfection for 20 hours. Mean values with standard deviations of 3 to 4 transfections per group, each with 6 to 12 replicate wells, are shown.

Comparing the constitutive levels of luciferase activity, Bartlett's test for homogeneity of variances gave a Bartlett statistic of 23.26, and a *P*-value of <0.0001, *i.e.* showing that the difference between the standard deviations of the four groups was highly significant. A non-parametric (Kruskal-Wallis) test was therefore used, which gave a Kruskal-Wallis statistic of 30.52, with three degrees of freedom, and a *P*-value of <0.001, *i.e.* showing a significant difference between the median values of the four groups. Dunn's Multiple Comparisons Test (a *post-hoc* comparison), comparing the results of each of the other groups of transfections versus the wild type (pL1A2N) gave a significant difference between SDM2 ($G_{-3595}T$) and wild type ($P<0.05$). On visual inspection of the data (Figure 4.8), it can be seen that the SDM2 group appears to have a higher mean luciferase activity than the pL1A2N group. However, the magnitude of this difference is small (0.72 relative light units per milliunit of β-galactosidase x 10^{-3}), giving a 1.2-fold increase in the mean value for the SDM2 group as compared to the pL1A2N group. It is possible that this translates into a significant functional difference *in vivo*, but unlikely.

Comparing the mean fold induction for each transfection experiment performed for the four plasmids using the Kruskal-Wallis test, gave a Kruskal-Wallis statistic of 4.99, and a *P*-value of 0.17. This means that there is not a significant difference between the fold induction in the four groups (mean induction 2.6-fold).

4.5 BAC clone generation and sequencing

4.5.1 Methods

Sequencing of *CYP1A2* positive BAC clones was undertaken in order to determine the sequence of the *CYP1A2* 5' flanking region further upstream than that previously published, in order to search for another XRE in this sequence.

A human bacterial artificial chromosome (BAC) library (Genome Systems Inc, St Louis, MO) was screened with a *Bam*HI/*Pst*I fragment (-808/-10, in intron 1). The probe was labelled by random priming with DNA polymerase I, large (Klenow) fragment using ^{32}P-dCTP (Ready To Go Kit, Pharmacia Biotech Inc., Piscataway, NJ). Labelled probe was added to membranes at 2.0 x 10^6 cpm/ml in standard hybridisation buffer, and hybridised at 42^{0}C overnight. Membranes were washed in 0.1xSSC, 0.5% sodium dodecyl sulphate, and exposed to autoradiographic film overnight at –70^{0}C. This procedure generated 3 positive BAC clones. These clones were then further probed with the following oligonucleotides: 5'-TCTGCCATCTTCTGCCTGGTATTCTG-3' (exon 2, positions 52-77), 5'-TCTTCCTCTTCCTGGCCATCCTGCTAC-3' (exon 7, positions 5238-5264), and 5'-TGGCAGAGCTCTTCCTCATGTGTGCAG-3' (5' flanking region, positions –1217 to -1245). Two out of the 3 clones were positive on all 3 hybridisations and were used in further studies (BAC7 and BAC8). The presence of *CYP1A2* in these clones was further confirmed by both PCR using primer pair cyp1abr2-cyp1abf and sequencing with primer M1. Clones BAC7 and BAC8 were then subject to direct sequencing using primer CYP1A2.5R (5'-AGCTCGATCATGTGTAGCTTG-3') and BigDye^{TM} Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer Applied Biosystems).

I designed a further primer, CYP1A2.5R2 (5'-CATCTTGCAGTGGTGTAAT-3') from the resultant sequence, and the clones were subject to direct sequencing with this primer.

4.5.2 Results of BAC clone sequencing

Sequencing of the cyp1abr2-cyp1abf PCR product from clones BAC7 and BAC8 with primer M1 was identical to the expected published sequence except for the $G_{-3649}C$ substitution and the T_{-3650} deletions already identified as above, thus confirming the presence of *CYP1A2* in these clones. Direct sequencing of BAC7 and BAC8 with CYP1A2.5R and CYP1A2.5R2 produced the sequence shown in Figure 4.9. Both sequences differed from that published by Quattrochi and Tukey (1989) in having an A_{-4072} deletion, and a C_{-4093} insertion (the latter shifting the *Kpn*I site further 5' by one nucleotide). I checked this region of pL1A2N (sequenced using primer M3), and found that this plasmid also had these sequence discrepancies compared with the original published sequence. The discrepancies therefore represent publication errors. I searched the 532 bp of novel sequence for XRE sites using MatInspector Version 2.2 (Quandt *et al.*, 1995), and found no XRE motif. However, blasting this sequence against sequence GENBANK accession number AF253322 ("Homo sapiens cytochrome P450 (CYP1A1) and cytochrome P450 (CYP1A2) genes, complete cds," submitted 06.04.2000 by Corchero *et al.*, and published in Corchero *et al.*, 2001) gave the result shown in Figure 4.10. Of note, there is a trinucleotide repeat, $(AAC)_6$ in my sequence at positions –4382 to –4399 (Figure 4.11), which is absent in the GENBANK sequence. This may represent a polymorphic site, or, alternatively, a sequencing error in the sequence reported by Corchero and colleagues, owing to the method of sequencing employed ("shotgun cloning"), and the likely instability of this sequence.

Similarly, there is a poly-T and a poly-A sequence at –4544 to –4550 and –4215 to –4221 respectively in my sequence (Figure 4.11), but not in the GENBANK sequence, which could again represent an artefact in the sequence of Corchero *et al.*, owing to the difficulty of sequencing across such regions and their method of sequencing, or these regions could be polymorphic. A variable length poly-T or poly-A sequence could have a functional consequence; there is a $(TA)_n$ repeat (n = 5-8) polymorphism in the UDP-glucuronosyltransferase 1 (*UGT1A1*) gene for which there is an inverse relationship between the number of TA repeats and promoter activity (Beutler *et al.*, 1998). Furthermore, comparison of the two sequences reveals three possible SNPs: -4597C/T, -4593C/A, -4583ΔC, and –4200A/G. None of these SNPs have been already deposited in dbSNP. Of these possible SNPs, as the –4200A/G site is an A in my sequence and an R (*i.e.* A/G) in the GENBANK sequence, this one may represent a true SNP. Interestingly, the -4072ΔA is confirmed in the GENBANK sequence. Further sequence analysis across this region is required to confirm the potentially polymorphic sites.

```
-4626     TGGGGTTCAAAGGATTCTCTTGCATCAGCCTCCTGAGTAGCTGGGACTACAGGCAT
-4570 GCGCCACCATGGTCGGCTAATTTTTTTGTATTTTTAGTAGAGATGGGGTTTTATCATGTT
-4510 GGCCAGGCTGGTCCAGAACTCCTGACCTCAAGTGATCCGCCCACATCAGCCTCCCAAAGT
-4450 GCTGAGATTACAGATGTGAGCCTCCACACCAGGCCTGAGACTCTGTCTCAAAACAACAAC
-4390 AACAACAACCAGAATGTATCAATATTCATCCACGAATTGTAACAAATATATTACACCACT
-4330 GCAAGATGTTAATAATAGGGGAAACTGCAGAGTGGGGGTGGTAAATGGCCACTTTTACCT
-4270 CCCTCATCATACTTTCCACTCAATTTTTCTGTGAACCAAAGACTGCTCTAAAAAAATCTA
-4210 TTAGCTTTTTAAAATTCCTTGGCTCCCCTCCAAAAAGTGTACATATGACATGATCTCATT
-4150 TATGTAAAATACAACAAGCAAAACAAATCCATGCAATAGATGTTGGGGTCATGGGTACCC
-4090 TTGAGAAAGGAACACAAC-GGGACTTCTTGGATG
                       A
```

Figure 4.9 Nucleotide sequence of the *CYP1A2* 5' flanking region further 5' than reported by Quattrochi and Tukey (1989). Sequence from –4626 to –4098 represents novel sequence. Discrepancies as compared with the sequence reported by Quattrochi and Tukey (1989) are shown: the C_{-4093} insertion is underlined, and the A_{-4072} deletion is illustrated.

```
Query: 30310 tggggttcaaaggattctcctgcctcagcctccctgagtagctgggactacaggcatgcg 30369
              |||||||||||||||||| ||| ||||||||| ||||||||||||||||||||||||
Sbjct: 1     tggggttcaaaggattctcttgcatcagcctcc-tgagtagctgggactacaggcatgcg 59


Query: 30370 ccaccatggtcggctaannnnnnngtatttttagtagagatggggttttatcatgttggc 30429
              |||||||||||||||||      ||||||||||||||||||||||||||||||||||||
Sbjct: 60    ccaccatggtcggctaattttttgtatttttagtagagatggggttttatcatgttggc 119


Query: 30430 caggctggtccagaactcctgacctcaagtgatccgcccacatcagcctcccaaagtgct 30489
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 120   caggctggtccagaactcctgacctcaagtgatccgcccacatcagcctcccaaagtgct 179


Query: 30490 gagattacagatgtgagcctccacaccaggcctgagactctgtctcaannnnnnnnnnnn 30549
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 180   gagattacagatgtgagcctccacaccaggcctgagactctgtctcaaaacaacaacaac 239


Query: 30550 nnnnnncagaatgtatcaatattcatccacgaattgtaacaaatatattacaccactgca 30609
                    ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 240   aacaaccagaatgtatcaatattcatccacgaattgtaacaaatatattacaccactgca 299


Query: 30610 agatgttaataataggggaaactgcagagtgggggtggtaaatggccacttttacctccc 30669
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 300   agatgttaataataggggaaactgcagagtgggggtggtaaatggccacttttacctccc 359


Query: 30670 tcatcatactttccactcaatttttctgtgaaccaaagactgctctnnnnnnntctatta 30729
              ||||||||||||||||||||||||||||||||||||||||||||       |||||||
Sbjct: 360   tcatcatactttccactcaatttttctgtgaaccaaagactgctctaaaaaaatctatta 419


Query: 30730 gctttttraaattccttggctcccctccaaaaagtgtacatatgacatgatctcatttat 30789
              ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 420   gctttttaaaattccttggctcccctccaaaaagtgtacatatgacatgatctcatttat 479


Query: 30790 gtaaaatacaacaagcaaaacaaatccatgcaatagatgttggggtcatgggtacccttg 30849
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 480   gtaaaatacaacaagcaaaacaaatccatgcaatagatgttggggtcatgggtacccttg 539


Query: 30850 agaaaggaacacaacgggacttcttggatg 30879
              ||||||||||||||||||||||||||||
Sbjct: 540   agaaaggaacacaacgggacttcttggatg 569
```

Figure 4.10 Pairwise blast ("Blast 2" from NCBI site) of gi:13430063 (AF253322) versus my novel 5' *CYP1A2* flanking sequence (25.10.02), identity = 93%, strand orientation plus/plus

```
-4626      TGGGGTTCAAAGGATTCTCYTGCMTCAGCCTCCCTGAGTAGCTGGGACTACAGGCAT
-4570 GCGCCACCATGGTCGGCTAATTTTTTTGTATTTTTAGTAGAGATGGGGTTTTATCATGTT
-4510 GGCCAGGCTGGTCCAGAACTCCTGACCTCAAGTGATCCGCCCACATCAGCCTCCCAAAGT
-4450 GCTGAGATTACAGATGTGAGCCTCCACACCAGGCCTGAGACTCTGTCTCAAAACAACAAC
-4390 AACAACAACCAGAATGTATCAATATTCATCCACGAATTGTAACAAATATATTACACCACT
-4330 GCAAGATGTTAATAATAGGGGAAACTGCAGAGTGGGGGTGGTAAATGGCCACTTTTACCT
-4270 CCCTCATCATACTTTCCACTCAATTTTTCTGTGAACCAAAGACTGCTCTAAAAAAATCTA
-4210 TTAGCTTTTTRAAATTCCTTGGCTCCCCTCCAAAAAGTGTACATATGACATGATCTCATT
-4150 TATGTAAAATACAACAAGCAAAACAAATCCATGCAATAGATGTTGGGGTCATGGGTACCC
-4090 TTGAGAAAGGAACACAACGGGACTTCTTGGATG
```

Figure 4.11 My *CYP1A2* 5' flanking novel sequence, with possible polymorphic sites underlined: trinucleotide repeat, $(AAC)_6$ at –4382 to –4399, a poly-T at –4544 to –4550 and a poly-A at –4215 to –4221, and three possible SNPs: -4597C/T, -4593C/A, -4583ΔC, and –4200A/G (conventional wobble codes given)

4.6 Discussion

I identified three novel SNPs in the *CYP1A2* 5' flanking region: a $T_{-3591}G$ substitution, a $G_{-3595}T$ substitution, and a T_{-3605} insertion. A PCR-RFLP test for the $T_{-3591}G$ polymorphism was designed and used to genotype 87 Caucasians, 104 African Americans, and 125 Taiwanese (Table 4.2); this revealed a significantly higher frequency of the G_{-3591} allele in Taiwanese as compared to Caucasians and African Americans.

The results of transfection experiments (Figure 4.8) showed that the SDM2 ($G_{-3595}T$) group had significantly higher constitutive activity than wild type, but only by a factor of 1.2. This small increase in promoter activity is unlikely to be of functional significance *in vivo*. Of note, if the $G_{-3595}T$ substitution were associated with increased promoter activity, the direction of effect would be in the opposite direction to that predicted by the work of Chang *et al.* (1997), whose data are consistent with the existence of a polymorphism causing reduced CYP1A2 activity present at a higher frequency in Taiwanese as compared with Caucasians. I performed a search for potential transcription factor binding sites in the region of interest using MatInspector Version 2.2, which showed that both the $T_{-3591}G$ and $G_{-3595}T$ polymorphisms lie within the putative binding site for CCAAT/enhancer binding protein β (C/EBPβ, -3582 to –3595 on forward strand). C/EBPβ is part of a family of transcription factors, that are widely expressed (Lekstrom-Himes and Xanthopoulos, 1998), may bind to a variety of sites including activating protein-1 (AP-1) sites, may heterodimerise with other transcription factors including c-*fos*, c-*jun*, and ATF-2, and has been shown to play a crucial role in the regulation of the expression of liver-specific genes (e.g. phosphoenolpyruvate carboxykinase, or PEPCK, Croniger *et al.*, 1998). It is of

possible interest that two of the polymorphisms ($T_{-3591}G$ and $G_{-3595}T$) that we have identified lie within the C/EBPβ binding site, and that the third one ($T_{-3605}ins$) is close.

Comparison of the TCDD-induced activity of the *CYP1A2* promoter in the four plasmids revealed no significant difference (mean induction 2.6-fold for the whole group). In the case of PEPCK regulation, C/EBP binding to a specific domain, P3(I), is required for the liver-specific (*i.e.* constitutive) expression of PEPCK, whereas its binding to 2 other domains, the cAMP regulatory element (CRE) and the glucocorticoid response unit (GRU) is involved in the induced expression. By analogy, it is therefore possible that this upstream C/EBPβ binding site is involved in the regulation of constitutive but not inducible *CYP1A2* expression, but, again, it is my opinion that a rigorous interpretation of my data (Section 4.4.3) does not support this.

The functional significance of the T_{-3605} insertion was not tested. However, although this lies within the putative binding sequence of a human transcription factor (Octamer factor 6, -3601 to –3614 on the forward strand or –3597 to –3613 on reverse strand), the T_{-3605} insertion does not alter its core recognition sequence. Given this fact, and the fact that the other two nearby polymorphisms do not appear to cause a significant functional alteration in *CYP1A2* promoter activity, in my opinion, it is also unlikely that this polymorphism would lead to a change in *CYP1A2* promoter activity. Nonetheless, even though these novel SNPs appear to be non-functional, they may be useful in disease association studies, provided that the interethnic variation in frequency is taken into account.

Genome walking and sequencing also revealed two other sequence discrepancies compared with the sequence published by Quattrochi and Tukey (1989): a $G_{-3649}C$ substitution and a T_{-3650} deletion. These sequence changes have since been reported by Nakajima *et al.* (1999). (In Fig 1 of Nakajima *et al.*, 1999, they have incorrectly given a C at their position –3650 in the sequence of Quattrochi and Tukey, 1989 – this should be a T.) Of note, Nakajima and colleagues also report a C_{-3484} deletion; I did not find this deletion in any of the samples sequenced. It is therefore possible that this represents a further polymorphic site, which is present in Japanese.

Sequencing of the BAC clone led to the description of 532 nucleotides of the 5' flanking region of *CYP1A2* further upstream than the sequence that was available at that time, in the process revealing 2 further errors in the Quattrochi and Tukey (1989) sequence, *i.e.* ΔA_{-4072} and a C_{-4093} insertion (the latter shifting the *Kpn*I recognition site upstream by one nucleotide). No XRE motif was found within the novel sequence, but comparison of this sequence with the GENBANK sequence in this region (AF253322) revealed differences between my novel sequence and the GENBANK sequence, including a poly-T, a poly-A, and a trinucleotide repeat sequence (all present in mine but not in the GENBANK sequence), and three possible SNPs. Whether or not these sequence discrepancies represent truly polymorphic sequence requires further investigation.

Three polymorphisms in *CYP1A2* which appear to be associated with a functional change have been reported since I performed my study: a $C_{-164}A$ substitution in intron 1 of Caucasians (allele *CYP1A2*1F*, Macleod *et al.*, 1998; Sachse *et al.*, 1999), a $C_{63}G$ substitution in exon 2 of Han Taiwanese (allele *CYP1A2*2*, Huang *et al.*, 1999), and a

$G_{-3858}A$ substitution in Japanese (allele *CYP1A2*1C*, Nakajima *et al.*, 1999). However, the *CYP1A2*1F* allele was only associated with a slightly higher inducibility in smokers when present in the homozygous state; the *CYP1A2*2* substitution was very rare in the population studied and its functional consequence was not investigated; and the *CYP1A2*1C* substitution only appeared to be associated with a reduction in CYP1A2 activity in smokers. In addition, a further report (Chida *et al.*, 1999) has described a T_{-2464} deletion (allele *CYP1A2*1D*, frequency 0.42) and a $T_{-740}G$ substitution in Japanese (allele *CYP1A2*1E*, frequency 0.082). The functional significance of these allelic variants has not been described. Chevalier *et al.* (2001) screened the 7 exons of *CYP1A2* including the 5' and 3' splice site consensus sequences in samples from 100 unrelated Caucasians using a PCR-SSCP strategy followed by selective sequencing. They identified 6 novel rare allelic variants: *CYP1A2*1G* (-740T>G, 1545T>C, 0.5%), *CYP1A2*1H* (951A>C, 1545T>C, 0.5%), *CYP1A2*3* (1042G>A, 1545T>C, 1%), *CYP1A2*4* (1156A>T, 0.5%), *CYP1A2*5* (1217G>A, 0.5%), *CYP1A2*6* (1291C>T, 0.5%). The last four variants include missense mutations, of which *CYP1A2*4* (I386F) encodes an amino substitution in the putative substrate recognition site. All of these *CYP1A2* polymorphisms reported to date are, however, together not sufficient to account for the apparent frequency of poor metabolisers seen in the bimodal distributions.

CYP1A2 phenotyping studies have varied in their conclusions as to whether the distributions support the existence of an underlying genetic component to CYP1A2 variability, and, although mathematical analysis of phenotyping methodology (Rostami-Hodjegan *et al.*, 1996) indicates that the ratio used by Kalow and Tang (1991a,b) should be the most discriminant of bimodality and independent of the renal

clearance of caffeine and paraxanthine, experimentally, only skewed unimodal or log normal distributions of this ratio have been observed (Kalow and Tang, 1991a, b; Vistisen *et al.*, 1992; Tang *et·al.*, 1994; Catteau *et al.*, 1995), except for the study of Schrenk *et al.* (1998), in which data best fitted by bimodal distributions in both smokers and non-smokers were observed.. The difference between the findings of Butler *et al.* (1992) and Kalow and Tang (1991a) on the one hand, and Chang *et al.* (1997) on the other hand may reflect failure to control for confounding variables such as various dietary factors (Lampe *et al.*, 2000).

Studies which have supported a bi- or tri-modal population distribution of CYP1A2 activity (Devonshire *et al.*, 1983; Butler *et al.*, 1992, Nakajima *et al.*, 1994) report a frequency of poor metabolisers of 10-14% in non-smokers, and a bimodal distribution also in smokers, suggesting a significant genetic impact both upon CYP1A2 activity, and upon CYP1A2 inducibility. The study of Schrenk *et al.* (1998) notably differs in the reported frequency of low CYP1A2 activity: 74% in male non-smokers and 80% in female non-smokers. With the metabolic ratio used by Butler *et al.* (1992) (urinary [1,7-dimethylurate + 1,7-dimethylaxanthine]/caffeine), 1,7-dimethylxanthine (also known as paraxanthine) is both a product and a substrate of CYP1A2, and therefore the time of sampling is critical, reproducibility for this method being optimal at 4-5 hours post dose (discussed by Kalow and Tang, 1993; Kadlubar *et al.*, 1996). This ratio is also sensitive to the renal clearance of caffeine and paraxanthine (Rostami-Hodjegan *et al.*, 1996). If a confounding factor such as renal clearance showed multimodality in its distribution, then this could lead to an erroneous conclusion that CYP1A2 is polymorphic. Indeed, a bimodal distribution of urine flow in some populations and interethnic variation in the renal clearance of dimethylxanthines has been suggested

(Tang *et al.*, 1994; Kalow & Tang, 1993). The ratio proposed by Fuhr and Rost (1994), *i.e.* the ratio of the plasma or salivary concentrations of paraxanthine to caffeine, is also subject to influence by the renal clearance of caffeine or paraxanthine, but, nonetheless, this ratio correlates highly ($r > 0.85$; 5-7 hour samples) with systemic caffeine clearance, and is hence regarded by Rostami-Hodjegan *et al.* (1996) as likely to be the "gold standard" for discrimination between CYP1A2 phenotypes, providing that there is enough separation between the mean enzyme activity of the different phenotypes and an adequate urine flow, similar in all subjects, is maintained.

In a recent CYP1A2 phenotyping study (Tantcheva-Poór *et al.*, 1999), using the salivary paraxanthine to caffeine ratio (which correlates highly with systemic caffeine clearance) in 786 Caucasians, the maximum likelihood test showed that the overall distribution of residuals was best described by the sum of two separate normal distributions, with 52% of subjects lying within the first normal distribution. Nonetheless, this apparent polymorphism could be equally well explained by non-specific deviation from the normal distribution, as evidenced by minor skew seen in the *P-P* plot. (The *P-P* plot was a plot of the cumulative probability of ANCOVA residuals to be expected assuming normal distribution versus the observed cumulative probability of the ANCOVA residuals, the ANCOVA being based on a simplified paraxanthine/caffeine ratio method for calculating caffeine clearance.) Indeed, the bimodal interpretation gives a poor metaboliser frequency of approximately 50%, substantially higher than that reported by earlier authors (Devonshire *et al.*, 1983; Butler *et al.*, 1992; Nakajima *et al.*, 1994), although not higher than the value reported by Schrenk *et al.* (1998). The difference between the poor metaboliser frequency in different studies may again be possibly due to confounding factors. Tantcheva-Póor *et*

al. (1999) analysed the contribution to the overall variance in CYP1A2 activity by a variety of covariates, and found that the following covariates accounted for 37% of the variance (estimated change relative to arbitrarily defined basal caffeine clearance, male, non-smoking, German resident): coffee (1.45-fold per litre of coffee drunk daily), body mass index (0.99-fold per kg m^{-2}), smoking (1.22-fold, 1.47-fold, 1.66-fold, and 1.72-fold for 1-5, 6-10, 11-20, and >20 cigarettes smoked per day), oral contraceptives (0.72-fold), country of residence (0.81-fold and 0.74-fold for Bulgaria and Slovakia), and female (0.90-fold).

As well as known environmental influences on CYP1A2 activity and intrinsic genetic variation, there are other possible causes of variation in CYP1A2 activity, such as gene-gene interactions. Coordinate regulation of *CYP1A2* and the UDP-glucuronyltransferase *UGT1.6* has been reported (Bock *et al.*, 1994). Macleod *et al.* (1997) reported that individuals possessing the glutathione transferase *GSTM1*0* allele had higher CYP1A2 activity for both non-induced and induced conditions, while individuals having the Ile/Ile *CYP1A1* genotype had higher CYP1A2 activity in the non-induced state, but lower CYP1A2 activity in the induced state than those with the Ile/Val genotype. In their studies of the colon carcinoma cell line LS180, Li *et al.* (1998) found that the concentration-response curves for induction by TCDD or 3-methylcholanthrene and the time courses of induction were very similar for CYP1A1, CYP1A2, and CYP1B1 (another enzyme of the CYP1 family, Shimada *et al.*, 1997), implying that the regulation of these enzymes may occur via a common pathway. Indeed, each member of the CYP1 family is inducible by TCDD via the arylhydrocarbon receptor (AhR) mechanism (Rowlands and Gustafsson, 1997), whereby the inducer plus AhR translocates to the nucleus, dimerises with the AhR

nuclear translocator (ARNT), and interacts with cis-acting elements termed xenobiotic responsive elements (XREs) in the 5' flanking region of the CYP1 gene. In mice, the genetic difference in susceptibility of different strains to TCDD-induced toxicity has been shown to correlate with polymorphism of the AhR (Nebert, 1989; Chang *et al.*, 1993). Polymorphisms in the human AhR have been identified (Nebert *et al.*, 1999; Garte and Sogawa, 1999), and it is possible that these may affect the inducibility of the CYP1 enzymes in man. In a study of quantitative trait loci affecting caffeine metabolism in inbred mouse strains, 3 loci were identified: one that colocalised with the murine CYP1A2 locus on chromosome 9 (suggesting the presence of an expression polymorphism affecting this gene), one on murine chromosome 1 (with a greater influence amongst males than females), and one on chromosome 4 (Casley *et al.*, 1999). This study demonstrates the possibility of genetic factors other than variation in CYP1A2 influencing the CYP1A2 activity, at least in mice.

There are deficiencies in the studies conducted to date that have searched for functional polymorphisms in *CYP1A2*. Nakajima *et al.* (1994) sequenced the 7 exons, exon-intron junctions, and the 5' flanking region to –3470 in only 5 individuals (of different phenotypes); in their later paper, Nakajima *et al.* (1999) sequenced –3418 to –4065 in the same 5 individuals; Welfare *et al.* (1999) performed screening by SSCP of the 7 exons, exon-intron boundaries, and 2 upstream regions in only 19 individuals (of different phenotypes), and sequenced only 2; Huang *et al.* (1999) sequenced the exons of only 8 Taiwanese (Han Chinese race); Sachse *et al.* (1999) only investigated the intron 1 polymorphism; and our screening focused on an upstream region, sequencing 20 Caucasians, 31 Taiwanese, and 10 African Americans. I conclude that firstly further phenotyping work is warranted in this field, *e.g.* use of the Kalow and Tang (1991a)

index of CYP1A2 activity in several different ethnic groups, carefully controlling for all possible confounding variables, and that, secondly, this should be combined with screening for functional polymorphisms in *CYP1A2* using sensitive methods such as denaturing high performance liquid chromatography (DHPLC, O'Donovan *et al.*, 1998), followed by PCR-sequencing as indicated, in a larger number of subjects than has yet been screened by this method (*e.g.* 50 from 3 different ethnic groups), and covering regions of *CYP1A2* that have as yet not been screened (including intronic sequences, where enhancers could reside). Furthermore, a recent report has indicated that *CYP1A1* and *CYP1A2* are separated by a 23 kb segment that contains no other open reading frames, and are orientated in opposite directions, so that the 5' flanking region is in common between the two genes (Corchero *et al.*, 2001, GenBank sequence accession number AF253322). Sequence analysis of this 5' flanking region revealed several XREs, in addition to the XREs previously reported (Fisher *et al.*, 1990; Kubota *et al.*, 1991; Quattrochi & Tukey, 1989), and it is possible that polymorphism in these could lead to differential inducibility of CYP1A2. Investigation of the possible coregulation of *CYP1A2* with *CYP1A1*, as well as the putative coregulation of *CYP1A2* with genes such as *CYP1B1*, the *UGT*s, and *GST*s, should prove fruitful.

CHAPTER FIVE

CLOZAPINE PHARMACOKINETICS AND PHARMACODYNAMICS STUDIED WITH CYP1A2-NULL MICE

5.1 Introduction

5.1.1 Clozapine pharmacokinetics

Clozapine is an atypical antipsychotic that is employed in the UK in cases of treatment-resistant schizophrenia or schizoaffective disorder. It is effective in approximately 30 to 60% of patients unresponsive to typical antipsychotics (Kane *et al.*, 1988; Kane, 1992), but the risk of agranulocytosis (0.7% in the first year of treatment, Atkin *et al.*, 1996) means that regular haematological monitoring is necessary and limits its use in the UK to treatment-resistant cases.

There is wide interindividual variation (approximately 10- to 50-fold) in the plasma levels of clozapine for a given dose (Perry *et al.*, 1991; Potkin *et al.*, 1994; Olesen *et al.*, 1995) and several studies indicate that clozapine concentrations of at least 350-420 μg/l are associated with clinical response (Perry *et al.*, 1991; Hasegawa *et al.*, 1993; Kronig *et al.*, 1995; Miller *et al.*, 1994; Potkin *et al.*, 1994). Clozapine is metabolised mainly in the liver, with the major metabolites being desmethylclozapine (or norclozapine) and clozapine *N*-oxide (Jann *et al.*, 1993; Figure 5.1). There have been several *in vitro* studies investigating the roles of the hepatic cytochromes in the generation of these metabolites. Eiermann and colleagues (1997) concluded that CYP1A2 and CYP3A4 were both involved in the demethylation of clozapine, and CYP3A4 in the *N*-oxidation. However, Linnet and Olesen (1997) suggested that

CYP2C19 and CYP3A4 would mediate 30% and 30-45% of clozapine metabolism respectively at therapeutic concentrations, with CYP1A2 mediating only about 10%. Tugnait *et al.* (1999) found that CYP1A2 and CYP3A4 both catalyse the demethylation and *N*-oxidation of clozapine, but that CYP1A2 played a more important role in the former and CYP3A4 in the latter.

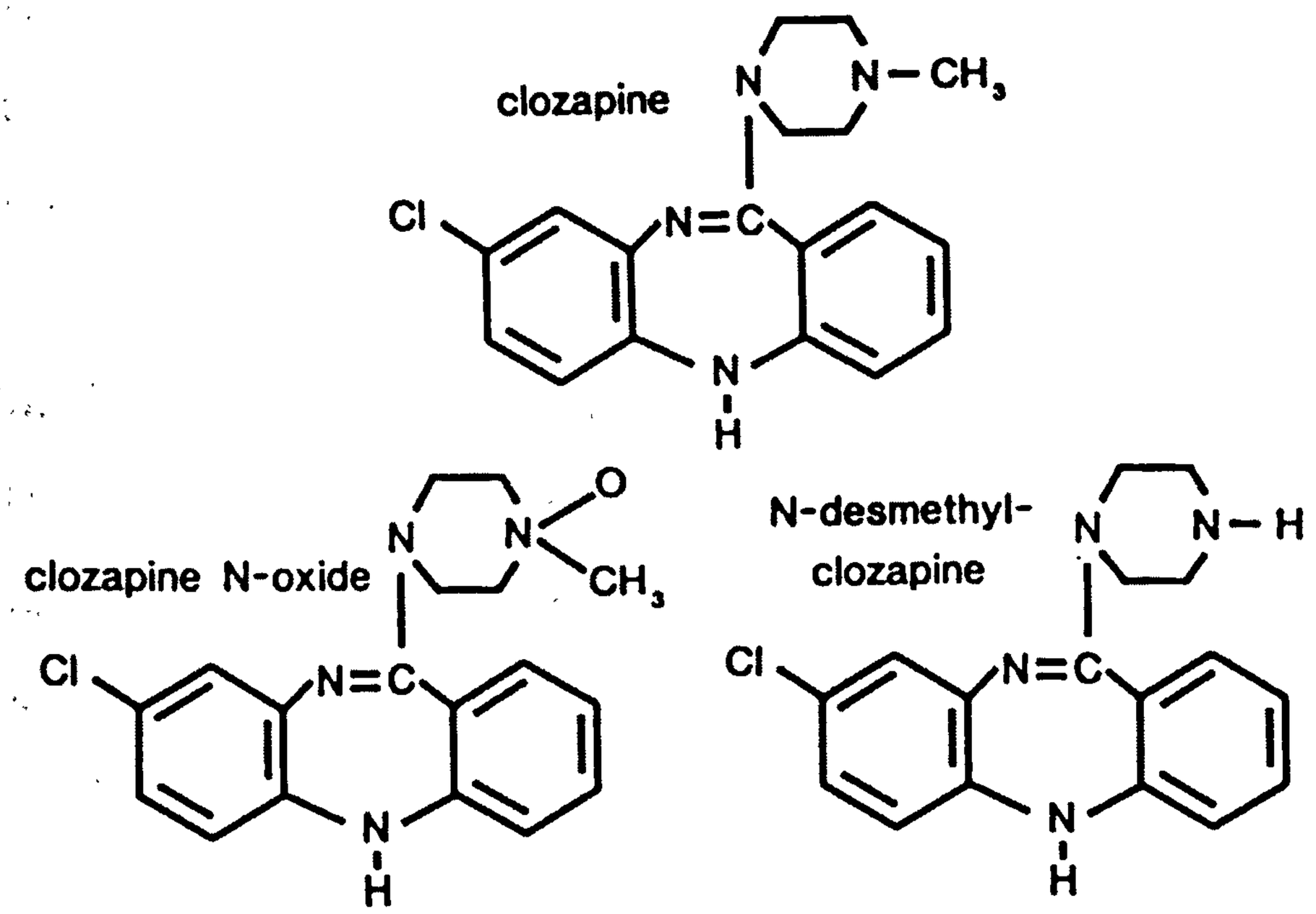


Figure 5.1 Structures of clozapine, clozapine *N*-oxide, and *N*-desmethylclozapine

CYP1A2 and CYP3A4 both show wide interindividual variation in activity (Aitchison *et al.*, 2000c). Jerling *et al.* (1997) studied the population pharmacokinetics of clozapine and found that the clearance of clozapine was distributed in a similar way to indices of CYP1A2 activity, indicating that CYP1A2 could be the major determinant of clozapine clearance. Most studies have demonstrated a trimodal or bimodal distribution to the pattern of CYP1A2 activity in a population (Section 4.6), with 5-14% of subjects in some studies (Butler *et al.*, 1992; Lang *et al.*, 1994; Nakajima *et al.*, 1994; Ou-Yang *et al.*, 2000), up to 50% in another (Tantcheva-Poór *et al.*, 1999), and up to 80% in one study (female non-smokers, Schrenk *et al.*, 1998) having relatively low CYP1A2 activity.

5.1.2 Human and mouse CYP1A2

Human and mouse CYP1A2 resemble each other closely in cDNA derived amino acid sequence (Kimura *et al.*, 1984; Jaiswal *et al.*, 1987) and in catalytic activity (Aoyama *et al.*, 1989). The human and mouse mRNA sequences are shown in Figure 5.2; there is 79% homology between them.

Figure 5.2 Human CYP1A2 mRNA sequence (GENBANK gi 20552470, top line) compared to mouse CYP1A2 mRNA sequence (GENBANK gi 6753565, below), showing regions of identity and amino acid sequence

```
95   gccacagagcttctcctggcctctgccatcttctgcctggtattctgggtgctcaagggt 154
     ||| ||||||| || |||||| |||||||||||||  | || |||||| || |||  |
28   gccccagagctgctactggccactgccatcttctgtttagtgttctggatggtcagagcc 87
10    A  P  E  L  L  L  A  T  A  I  F  C  L  V  F  W  M  V  R  A

155  ttgaggcctcgggtccccaaaggcctgaaaagtccaccagagccatggggctggcccttg 214
     |  ||| | | ||| |||||||||||||| | |||||| |  || ||||||| ||||||
88   tcaaggacccaggttcccaaaggcctgaagaatccacccggaccctggggcttgcccttc 147
30    S  R  T  Q  V  P  K  G  L  K  N  P  P  G  P  W  G  L  P  F

215  ctcgggcatgtgctgaccctggggaagaacccgcacctggcactgtcaaggatgagccag 274
     | |||||  ||||||| |||||||||||||| |||||| ||||| || || |||| |||
148  attgggcacatgctgactgtggggaagaacccacacctgtcactgacacggctgagtcag 207
50    I  G  H  M  L  T  V  G  K  N  P  H  L  S  L  T  R  L  S  Q

275  cgctacggggacgtcctgcagatccgcattggctccacgcccgtgctggtgctgagccgc 334
     |  || |||||||| |||||||||||||| |||||||| || ||| |||||||||||  ||
208  cagtatggggacgtgctgcagatccgcatcggctccactcctgtggtggtgctgagcggc 267
70    Q  Y  G  D  V  L  Q  I  R  I  G  S  T  P  V  V  V  L  S  G

335  ctggacaccatccggcaggccctggtgcggcagggcgacgatttcaagggccggcctgan 394
     |||  |||||||| |||||||||||| ||||||| || || |||||||||| || ||
268  ctgaacaccatcaagcaggccctggtgaggcagggagatgacttcaagggccgaccagac 327
90    L  N  T  I  K  Q  A  L  V  R  Q  G  D  D  F  K  G  R  P  D

395  ccntnctnacancctnccaccctcatcactgatggccagagcttgaccttcagcacagac 454
     |  |    |||  |  |  ||| || ||||||  | ||| |||||  ||||  ||||  | |||||
328  ctct----acagctt--cacacttatcactaacggcaagagcatgactttcaacccagac 381
110   L      Y  S  F    T  L  I  T  N  G  K  S  M  T  F  N  P  D

455  tctggaccggtgtgggctgcccgccggcgcctggcccagaatgccctcaacaccttctcc 514
     ||||||||  |||||||||||||||||||||||||||||||||  |||||||  ||  |  |||||||
382  tctggacccgtgtgggctgcccgccggcgcctggcccaggatgccctgaagagcttctcc 441
128   S  G  P  V  W  A  A  R  R  R  L  A  Q  D  A  L  K  S  F  S

515  atcgcctctgacccagcttcctcatcctcctgctacctggaggagcatgtgagcaaggag 574
     ||  |||||  |||||  |  ||  |||||||  |||||  |||||||||| ||||||||||||
442  atagcctcggacccgacgtcagcatcctcttgctatttggaggagcacgtgagcaaggag 501
148   I  A  S  D  P  T  S  A  S  S  C  Y  L  E  E  H  V  S  K  E

575  gctaaggccctgatcagcaggttgcaggagctgatggcagggcctgggcacttcgaccct 634
     |||||    ||  |||||| | | ||| ||  |||||||| |  ||| |||||||| ||
502  gctaaccatctcgtcagcaagcttcagaaggcgatggcagaggttggccacttcgaacca 561
168   A  N  H  L  V  S  K  L  Q  K  A  M  A  E  V  G  H  F  E  P

635  tacaatcaggtggtggtgtcagtggccaacgtcattggtgccatgtgcttcggacagcac 694
       ||  ||||||||||  ||  |||||  |||||||||||||||||||||||  ||  || ||
562  gtcagccaggtggtggaatcggtggctaacgtcattggtgccatgtgctttgggaagaac 621
188   V  S  Q  V  V  E  S  V  A  N  V  I  G  A  M  C  F  G  K  N

695  ttccctgagagtagcgatgagatgctcagcctcgtgaagaacactcatgagttcgtggag 754
     |||||   ||  |||||  |||||||| | | |||||||  ||||   | ||  || ||||||
622  ttcccccggaagagcgaggagatgctgaacatcgtgaataacagcaaggactttgtggag 681
208   F  P  R  K  S  E  E  M  L  N  I  V  N  N  S  K  D  F  V  E
```

```
755  actgcctcctccgggaacccccctggacttcttccccatccttcgctacctgcctaaccct 814
     |  ||  |  ||||  |||||   |   ||||||||||||||   ||||  ||||||||||||  |||||
682  aatgtcacctcagggaatgcagtggacttcttcccggtcctgcgctacctgcccaacccg 741
228   N   V   T   S   G   N   A   V   D   F   F   P   V   L   R   Y   L   P   N   P

815  gccctgcagaggttcaaggccttcaaccagaggttcctgtggttcctgcagaaaacagtc 874
     |||||  |||||||  |||  |||||||   |  |   |||  ||   |||  ||||||||||||  |||
742  gccctcaagaggtttaagaccttcaatgataacttcgtgctgtttctgcagaaaactgtc 801
248   A   L   K   R   F   K   T   F   N   D   N   F   V   L   F   L   Q   K   T   V

875  caggagcactatcaggactttgacaagaacagtgtccgggacatcacgggtgccctgttc 934
     ||||||||||||  ||  |||||  ||||||||||  |||   ||||||||  ||||||||||||
802  caggagcactaccaagacttcaacaagaacagtatccaagacatcacaagtgccctgttc 861
268   Q   E   H   Y   Q   D   F   N   K   N   S   I   Q   D   I   T   S   A   L   F

935  aagcacagcaagaaggggcctagagccagcggcaacctcatcccacaggagaagattgtc 994
     |||||||||  ||||       |  ||  ||  |||   ||||||||  ||||||||||||||
862  aagcacagcgagaa---ctacaaagacaatggcggtctcatccccgaggagaagattgtc 918
288   K   H   S   E   N       Y   K   D   N   G   G   L   I   P   E   E   K   I   V

995  aaccttgtcaatgacatctttggagcaggatttgacacagtcaccacagccatctcctgg 1054
     |||  ||||||||||||||||||||||  ||  ||||||||||||||||||||||||  |||||
919  aacattgtcaatgacatctttggagctggctttgacacagtcaccacagccatcacctgg 978
307   N   I   V   N   D   I   F   G   A   G   F   D   T   V   T   T   A   I   T   W

1055 agcctcatgtaccttgtgaccaagcctgagatacagaggaagatccagaaggagctggac 1114
     |||  |  ||   ||||||||   ||||  |  |  ||||||||||||||  ||||||||||
979  agcattttgctacttgtgacatggcctaacgtgcagaggaagatccatgaggagctggac 1038
327   S   I   L   L   L   V   T   W   P   N   V   Q   R   K   I   H   E   E   L   D

1115 actgtgattggcagggagcggcggccccggctctctgacagaccccagctgccctacttg 1174
     ||  |||  ||||||||||  ||||  ||  |||||  ||||||  |  ||||||||||  ||  |
1039 acggtggttggcagggatcggcaaccacggctttctgaccgtccccagctgccatatcta 1098
347   T   V   V   G   R   D   R   Q   P   R   L   S   D   R   P   Q   L   P   Y   L

1175 gaggccttcatcctggagaccttccgacactcctccttcttgcccttcnnaccatccccc 1234
     ||||||||||||||||||||  ||  ||||  ||  |  |||||   |  ||||||  ||||||||||
1099 gaggccttcatcctggagatctaccgatacacatcctttgtccccttc--accatccccc 1156
367   E   A   F   I   L   E   I   Y   R   Y   T   S   F   V   P   F     T   I   P

1235 acagcacaacaagggacacaacgctgaatggcttctacatccccaagaaatgctgtgtct 1294
     ||||||||||  ||||||||   |  ||||||||||||  ||||  ||||||  |   |||||  |||
1157 acagcacaacgagggacacctcactgaatggcttccacattcccaaggagcgctgtatct 1216
386   H   S   T   T   R   D   T   S   L   N   G   F   H   I   P   K   E   R   C   I

1295 tcgtaaaccagtggcaggtcaaccatgacccagagctgtgggaggacccctctgagttcc 1354
     |  ||||||||||||||||||||||||||||     |||  ||||  |  |||||||  ||  |||||
1217 acataaaccagtggcaggtcaaccatgatgagaagcagtggaaagacccctttgtgttcc 1276
406   Y   I   N   Q   W   Q   V   N   H   D   E   K   Q   W   K   D   P   F   V   F

1355 ggcctgagcggttcctcaccgccgatggcactgccattaacaagcccttgagtgagaaga 1414
     |  ||  ||||||||  ||  |||    |    |  |  |||||  |||||  ||   |||  ||||||
1277 gcccagagcggtttcttaccaataacaactcggccatcgacaagacccagagcgagaagg 1336
426   R   P   E   R   F   L   T   N   N   N   S   A   I   D   K   T   Q   S   E   K

1415 tgatgctgtttggcatgggcaagcgccggtgtatcggggaagtcctggccaagtgggaga 1474
     |||||||  ||  |||  ||||  ||||||||||||  ||  |||||   |||  ||||||||||||
1337 tgatgctcttcggcttgggaaagcgccggtgcattggggagatcccggccaagtgggaag 1396
446   V   M   L   F   G   L   G   K   R   R   C   I   G   E   I   P   A   K   W   E

1475 tcttcctcttcctggccatcctgctacagcaactggagttcagcgtgccgccgggcngtg 1534
     ||||||||||  |  ||||||||||  |||||  ||||||||  ||  |||||  |||||   |||
1397 tcttcctcttcttagccatcctgctgcagcatctggagtttagtgtgccaccggg-tgtg 1455
466   V   F   L   F   L   A   I   L   L   Q   H   L   E   F   S   V   P   P   G     V
```

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1535 aaagtcgacctgacccccatctacgggctgaccatgaagcacgcccgctgtgaacatgtc 1594
     || || |||||||| |||| ||| ||| |||||||||||| ||    ||||||||| |||
1456 aaggtggacctgacacccaactatgggttgaccatgaagcccgggacctgtgaacacgtc 1515
486   K  V  D  L  T  P  N  Y  G  L  T  M  K  P  G  T  C  E  H  V


1595 caggcgcggctgcgcttctcca 1616
     |||||  |||  ||||| ||||
1516 caggcatggccacgcttttcca 1537
506   Q  A  W  P  R  F  S
```

5.1.3 Aims of this study

Given the inconsistencies in the conclusions of the above studies regarding the contribution of CYP1A2 to clozapine metabolism, my aim was to use the CYP1A2 -/- (null) mouse in order to investigate the *in vivo* contribution of CYP1A2 to clozapine pharmacokinetics. In addition, I used the CYP1A2 -/- mouse as a model for low CYP1A2 activity in man, through the use of behavioural ratings aiming to draw conclusions regarding the pharmacodynamic effects of clozapine in individuals relatively deficient in CYP1A2 activity.

5.2 Methods

The methodology for this Chapter is given in Chapter 2: Sections 2.2.10.1 (Study design), 2.1.3 (Mice), 2.2.10.3 (HPLC analysis), 2.2.10.2 (Behavioural effects ratings), and 2.3.6 (Statistical analysis). All the data reported in this Chapter have also already been published (Aitchison *et al.*, 2000b).

5.3 Results

5.3.1 Pharmacokinetic parameters

The mean weights (± standard deviations) of the wild-type (N = 4) and CYP1A2 -/- mice (N = 4) were 23.28 g (± 3.21) and 27.06 g (± 3.27) respectively. The Mann-

Whitney U test revealed a 1-tailed *P* value of 0.17, *i.e.* the two groups did not differ significantly in their mean weights.

Table 5.1 gives the pharmacokinetic parameters of clozapine, desmethylclozapine, and clozapine *N*-oxide in the 2 groups of mice, and Figures 5.3 to 5.5 show the concentrations of clozapine, desmethylclozapine, and clozapine *N*-oxide versus time. The area under the concentration-time curve (AUC) of clozapine was 2.6 times greater, the clearance of clozapine was 2.6 times slower, and the half-life was 1.2 times longer in the CYP1A2 -/- mice as compared to the wild-type mice. Sixty-one percent of the clozapine clearance in wild-type mice is mediated by CYP1A2 (calculated by the formula given in Section 2.3.6). For desmethylclozapine, the AUC was 1.6 times lower in the CYP1A2 -/- mice as compared to the wild-type mice, while for clozapine *N*-oxide, although the AUC was 1.4 times greater in the CYP1A2 -/- mice, this difference did not quite reach statistical significance ($P = 0.0571$).

5.3.2 Behavioural effects

The behavioural effects of clozapine on the wild-type and CYP1A2 -/- mice are shown in Figures 5.6 and 5.7. The CYP1A2 -/- mice were significantly more drowsy and showed more motor impairment than the wild-type mice ($P = 0.0145$ in both instances, Mann-Whitney U test). In addition, myoclonus was noted in one wild-type mouse at 240 min, in two CYP1A2 -/- mice at 60 min, in one CYP1A2 -/- mouse at 120 min, and in one CYP1A2 -/- mouse at 120 min and at 240 min.

Table 5.1 Pharmacokinetic parameters of clozapine, desmethylclozapine, and clozapine *N*-oxide in male wild-type and CYP1A2 -/- mice after a single 10 mg/kg intraperitoneal dose of clozapine (mean ± SD)

	Wild-type ($n = 4$)	CYP1A2 -/- ($n = 4$)
Clozapine $AUC_{0\rightarrow\infty}$ (mg min ml^{-1})	42.9 ± 3.9	111.3 ± 11.1**
Clozapine clearance/F (ml kg^{-1} min^{-1})	0.234 ± 0.020	0.091 ± 0.009**
Clozapine half-life (min)	110.1 ± 8.1	129.8 ± 3.1**
Desmethylclozapine $AUC_{0\rightarrow\infty}$ (mg min ml^{-1})	11.9 ± 2.7	7.6 ± 2.2*
Clozapine *N*-oxide $AUC_{0\rightarrow\infty}$ (mg min ml^{-1})	2.0 ± 0.2	2.7 ± 0.8

*1-tailed $P = 0.0286$, **1-tailed $P = 0.0143$ (Mann-Whitney U test).

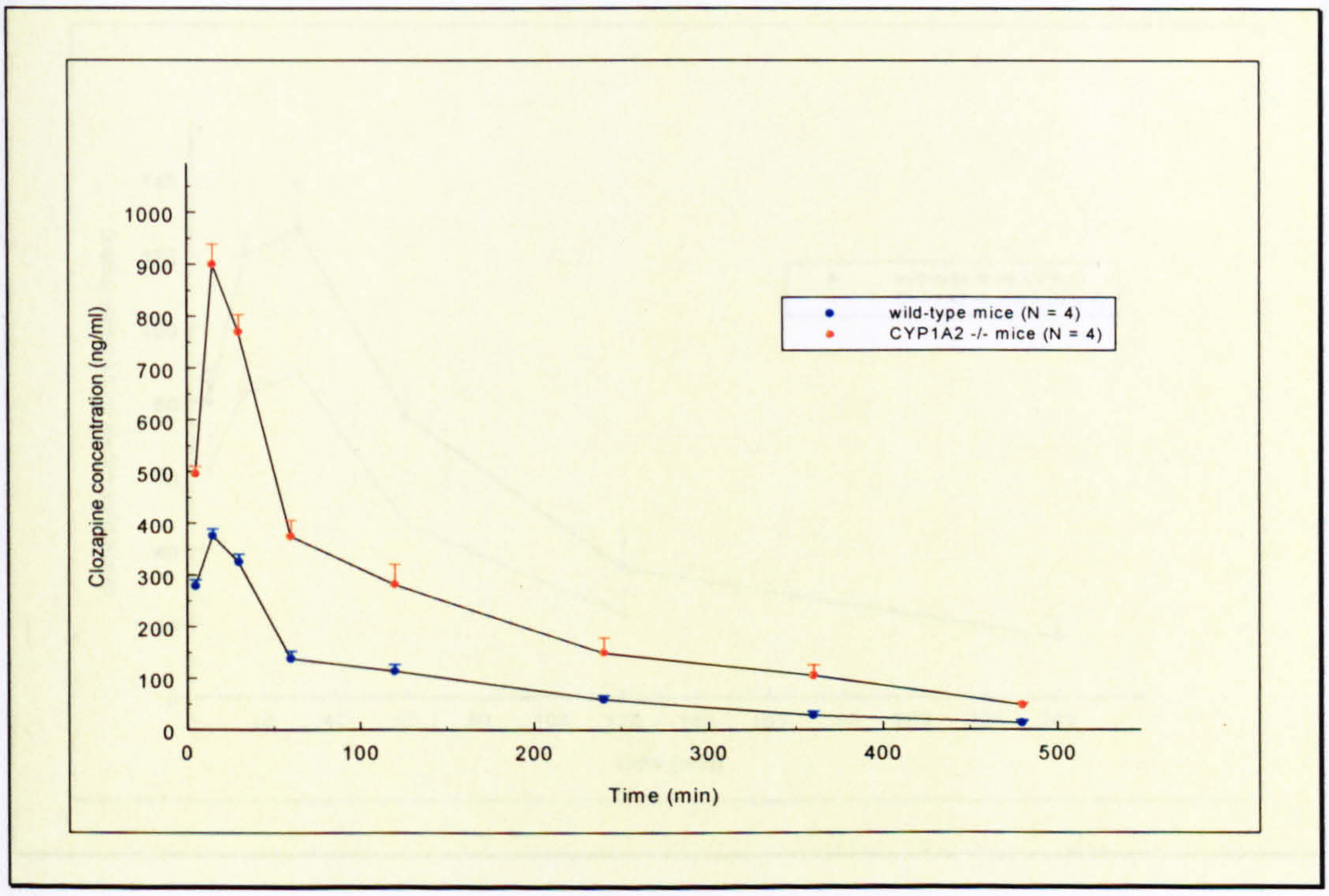

Figure 5.3 Whole blood clozapine concentration-time curves after a 10 mg/kg intraperitoneal dose of clozapine to male wild-type and *CYP1A2* -/- mice, administered at time 0. Mean values + SD are given.

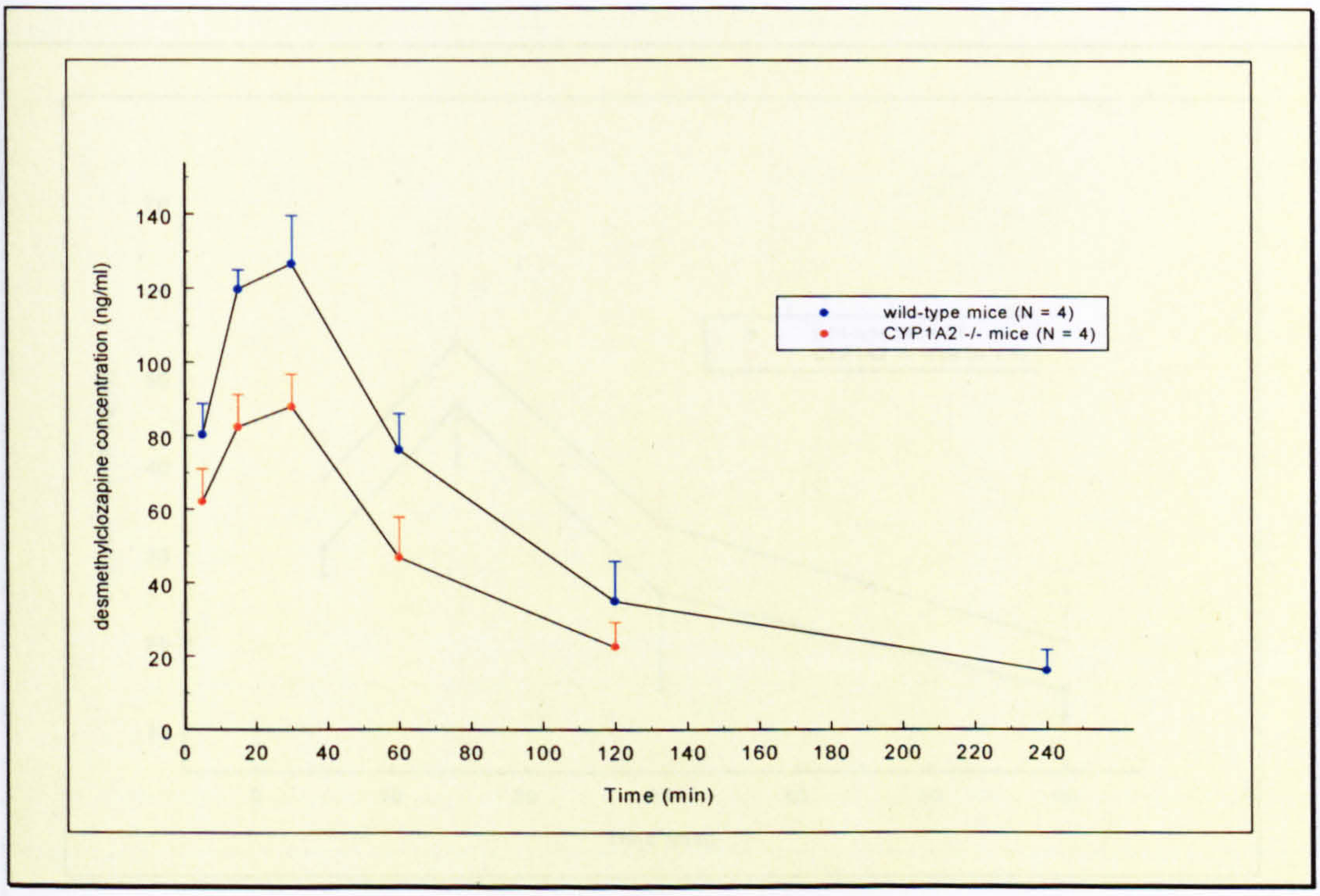

Figure 5.4 Whole blood desmethylclozapine concentration-time curves after the 10 mg/kg intraperitoneal dose of clozapine. For the wild-type mice at 240 min, the mean of 2 values is reported (the other 2 values were below the limit of detection, *i.e.* less than 5.0 ng/ml), otherwise the data are mean of 4 values + SDs.

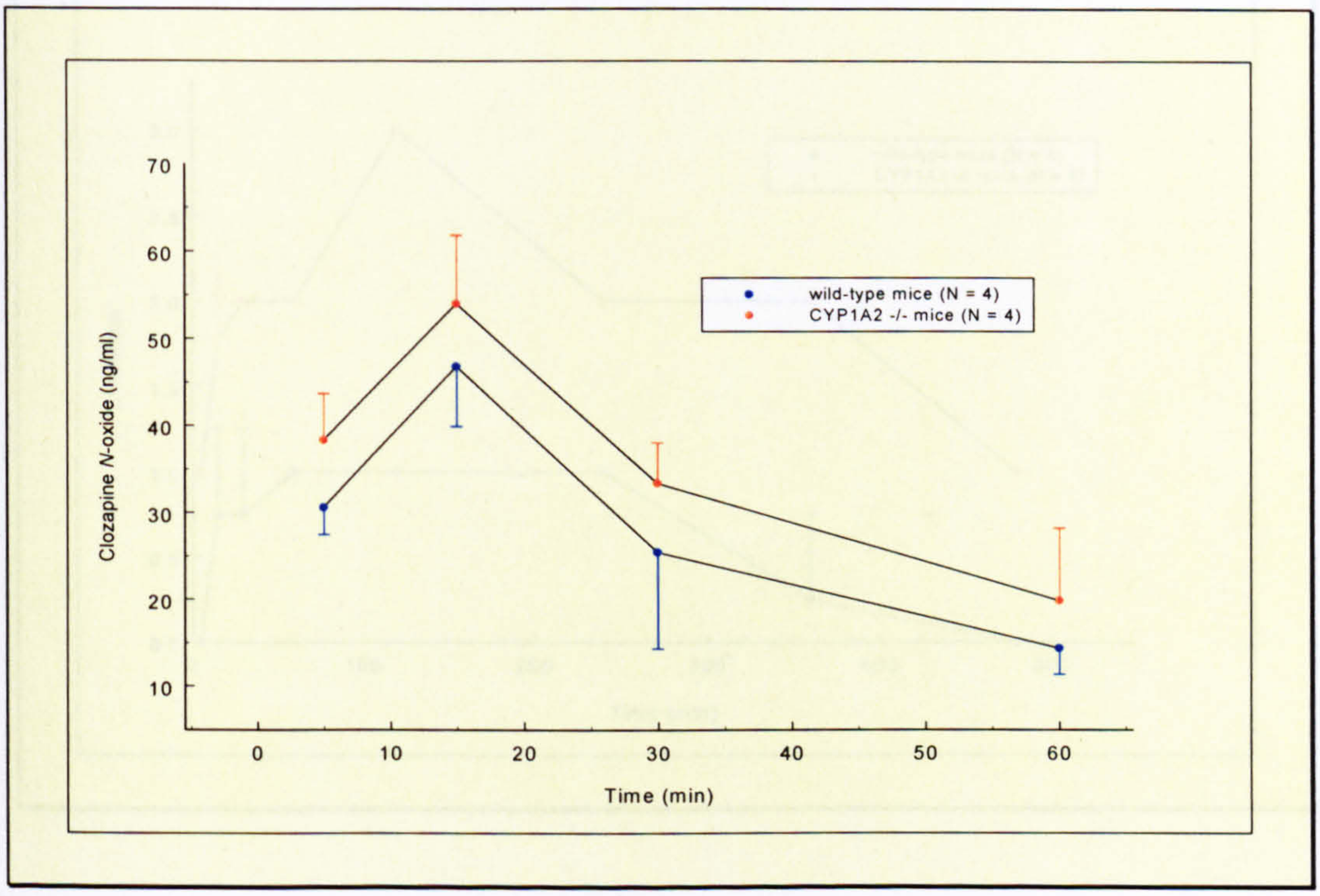

Figure 5.5 Whole blood clozapine *N*-oxide concentration-time curves (mean ± SD) after the10 mg/kg intraperitoneal dose of clozapine. Values beyond time = 60 min for both wild-type and CYP1A2 -/- mice were below the limit of detection (5.0 ng/ml).

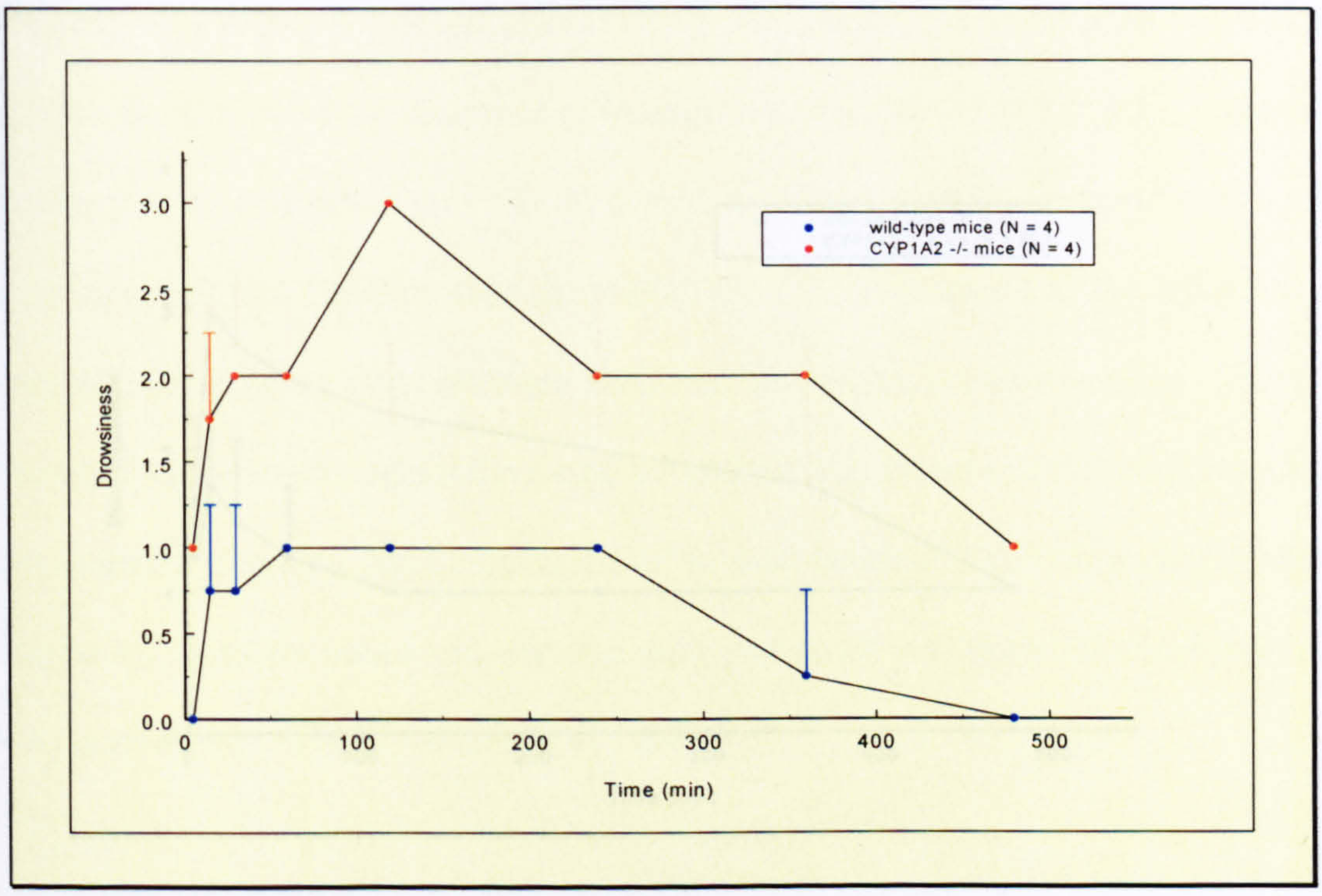

Figure 5.6 Degree of drowsiness versus time after the 10 mg/kg intraperitoneal dose of clozapine. Mice were rated for drowsiness using the criteria given in Table 2.3. Mean scores + SD are given.

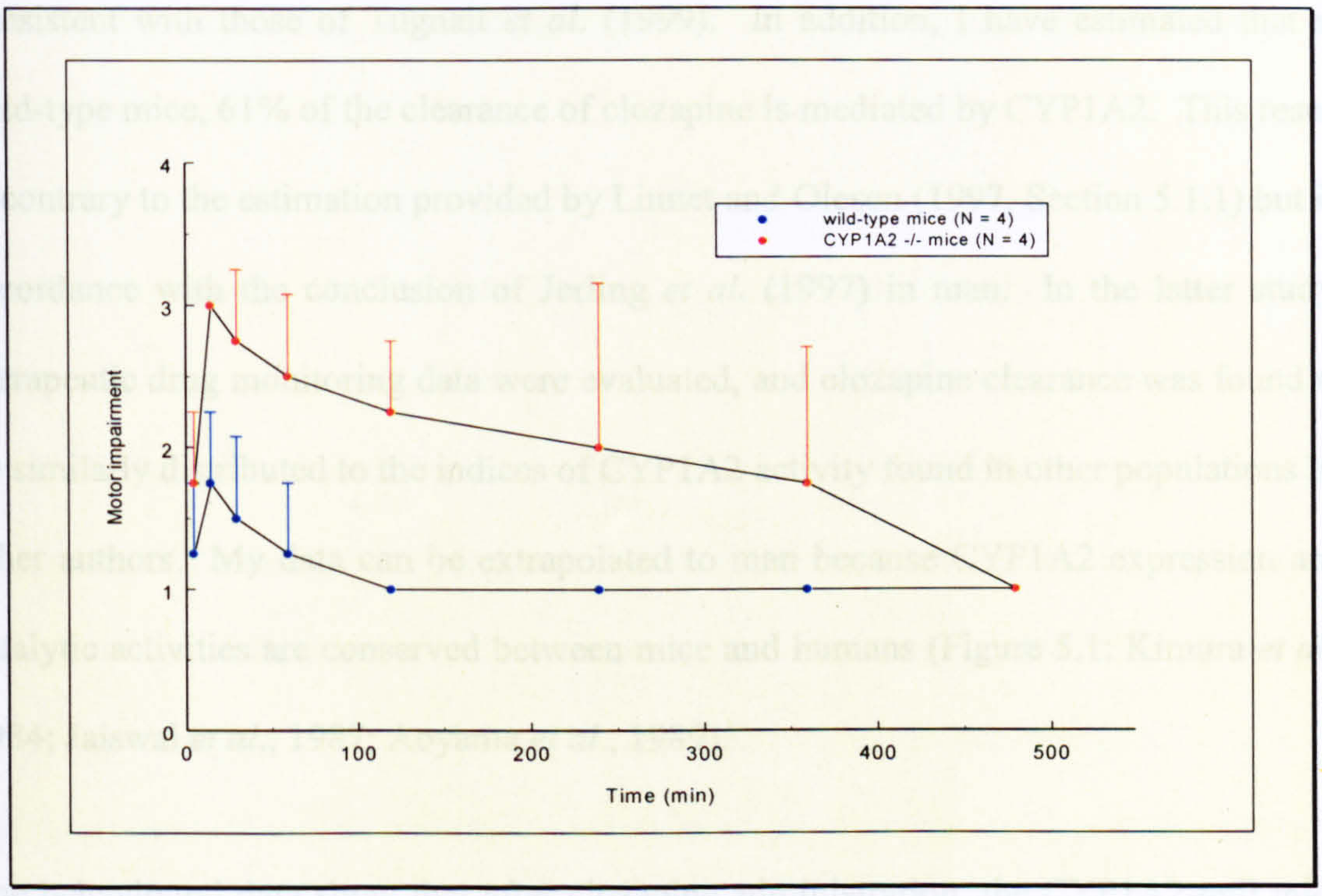

Figure 5.7 Degree of motor impairment versus time after the 10 mg/kg intraperitoneal dose of clozapine. Motor impairment in the mice was rated using the criteria given in Table 2.3. Mean scores + SD are given.

5.4 Discussion

My results indicate that CYP1A2 contributes significantly to the demethylation of clozapine *in vivo*. The finding of a slightly greater AUC for clozapine *N*-oxide in the CYP1A2 -/- mice may be explained by the following: in the absence of CYP1A2, with a consequent higher concentration of clozapine, more clozapine was available to undergo *N*-oxidation by enzymes other than CYP1A2 (which might have included other enzymes, such as CYP3A4, possibly being induced by clozapine). If CYP1A2 contributes to the *N*-oxidation of clozapine in mice, it must do so only to a very minor degree. These findings confirm the *in vitro* results of Eiermann *et al.* (1997) and are

consistent with those of Tugnait *et al.* (1999). In addition, I have estimated that in wild-type mice, 61% of the clearance of clozapine is mediated by CYP1A2. This result is contrary to the estimation provided by Linnet and Olesen (1997, Section 5.1.1) but in accordance with the conclusion of Jerling *et al.* (1997) in man. In the latter study, therapeutic drug monitoring data were evaluated, and clozapine clearance was found to be similarly distributed to the indices of CYP1A2 activity found in other populations by other authors. My data can be extrapolated to man because CYP1A2 expression and catalytic activities are conserved between mice and humans (Figure 5.1; Kimura *et al.*, 1984; Jaiswal *et al.*, 1987; Aoyama *et al.*, 1989).

The behavioural data show that after clozapine administration, the CYP1A2-null mice were significantly more drowsy, showed more motor impairment, and had more myoclonus than the wild-type mice. Clinical data indicate that sedation is the most commonly reported adverse effect of clozapine, occurring in 39% of patients (Safferman *et al.*, 1991). My data predict that for a given dose of clozapine, individuals with relatively low CYP1A2 activity might show a greater degree of sedation than extensive metabolisers (EMs). Myoclonus occurs in 2% of patients on clozapine (Lieberman and Safferman, 1992) and may progress to generalised seizure activity (Berman *et al.*, 1992; Gouzoulas *et al.*, 1993; Meltzer and Ranjan 1994). Seizures are a dose-related adverse effect, the frequency of seizures at clozapine doses of less than 300 mg/day, 300-600 mg/day, and above 600 mg/day being 1%, 2.7%, and 4.4% respectively (Devinsky *et al.*, 1991). My data similarly indicate that CYP1A2 low activity individuals might be more prone to myoclonus, and therefore might be at greater risk of generalised seizure activity for a given dose of clozapine. The risk of this and other dose-related adverse effects could be minimised by titrating the dose up

more slowly than usual, monitoring carefully for the emergence of effects such as sedation.

The fact that the CYP1A2 -/- mice had significantly higher levels of clozapine would also imply that individuals relatively deficient in CYP1A2 would respond at lower doses of clozapine than those with higher CYP1A2 activity. The dose of clozapine employed in this study (10 mg/kg) was chosen as the results of preliminary experiments indicated that this dose was necessary to achieve informative data for desmethylclozapine and clozapine *N*-oxide, and would be equivalent to a dose of approximately 700 mg or 600 mg clozapine in an average man or woman respectively, as is demonstrated by the fact that the peak serum level in the wild-type mice (300-400 ng/ml) is in the therapeutic treatment range for clozapine in man. In a study of 12,760 clozapine recipients in the UK and Ireland, the mean clozapine dose after 12 weeks of treatment was 388 mg/day, the mean maximum dose was 462 mg/day, and 41% had a peak dose of more than 500 mg/day (Munro *et al.*, 1999). My study indicates that daily doses of greater than 600-700 mg (especially as a single dose) should not be used in individuals with relatively low CYP1A2 activity.

Parallels may be drawn between my study and studies investigating the correlation between adverse effects of typical antipsychotics and CYP2D6 metaboliser status. In a single dose pharmacokinetic study of perphenazine administered to six CYP2D6 PMs and six CYP2D6 EMs, the PMs reported more adverse effects, especially tiredness (Dahl-Puustinen *et al.*, 1989). Similarly, in a study investigating severe adverse effects during the first days of treatment with a phenothiazine or haloperidol, CYP2D6 PMs had a higher incidence of concentration-dependent effects, such as oversedation,

postural hypotension and autonomic effects (Spina *et al.*, 1992). However, concentration-dependent effects tend to reduce with time (Aitchison *et al.*, 1999c); in the case of clozapine, patients usually develop tolerance to the sedative effects within 4 to 6 weeks of treatment (Marinkovic *et al.*, 1994). Correlations between metaboliser status and concentration-dependent effects are therefore most relevant to the initial, dose-finding stages of prescribing.

Other adverse effects of clozapine, such as neutropenia and agranulocytosis, do not appear to be concentration-dependent. The peak incidence of neutropenia and agranulocytosis occurs within the first 6-18 weeks of clozapine treatment, being 1.27% and 0.7% respectively, and the risk of both decreases with increases in clozapine dose (Munro *et al.*, 1999). The pathophysiological mechanism of agranulocytosis is uncertain, but hypotheses include a direct toxic effect on neutrophils or haemopoietic precursors (Veys *et al.*, 1992; Gerson *et al.*, 1994, Deliliers *et al.*, 1998), and an immunological basis (Pisciottta *et al.*, 1992), the latter with a possible genetic predisposition (Corzo *et al.*, 1995; Lieberman *et al.*, 1990). Desmethylclozapine at high concentrations has been seen to have a toxic effect on the precursors of both myeloid and erythroid lineages (Gerson *et al.*, 1994), and the desmethylclozapine/clozapine ratio has been seen to be inversely correlated with neutrophil count in patients treated with clozapine (Mauri *et al.*, 1998). If the formation of desmethylclozapine is primarily CYP1A2-dependent, then the desmethylclozapine/clozapine ratio should reflect CYP1A2 activity. This would imply that individuals with relatively high CYP1A2 activity could be at greater risk of clozapine-induced neutropenia and agranulocytosis. However, other studies have failed to find an association between desmethylclozapine levels or

desmethylclozapine/clozapine ratio and granulocyte counts (Hasegawa *et al.*, 1994; Combs *et al.*, 1997).

Another hypothesis regarding the mechanism of agranulocytosis is that it involves metabolic activation of clozapine with the formation of free radicals which are able to bind covalently to neutrophil or bone marrow proteins, then leading to agranulocytosis either by a direct toxic (analagous to the covalent binding of acetaminophen in the liver, Pumford and Halmes, 1997) or an immunological route (Fischer *et al.*, 1991; Pirmohamed *et al.*, 1995; Liu and Uetrecht , 1995; Maggs *et al.*, 1995; Gardner *et al.*, 1998a and 1998b). Chemically reactive metabolites of clozapine may be formed by neutrophil myeloperoxidase (Gardner *et al.*, 1998a) or by cytochrome P450s CYP3A, CYP2C9, and CYP2E1 (Pirmohamed *et al.*, 1995). Cytochrome P450 isoforms appear to be expressed not only in the liver, but also in neutrophils and bone marrow stem cells (Murray *et al.*, 1988; Gonzalez, 1992). The formation of the reactive metabolites catalysed by the P450s is reversible (Pirmohamed *et al.*, 1995); under conditions of relatively low CYP1A2 activity, the increase in clozapine concentration would be expected to lead to an increase in the proportion of clozapine passing down the pathway of reactive metabolite formation. If this hypothesis were correct, then relatively low CYP1A2 activity might be expected to be associated with agranulocytosis. However, agranulocytosis and CYP1A2 activity do not show the same ethnic stratification, *i.e.* agranulocytosis occurs at a greater frequency in Asians but not Orientals or African-Caribbeans compared with Caucasians (Munro *et al.*, 1999), while CYP1A2 activity appears to be lower in Orientals and Blacks (Le Marchand *et al.*, 1997; Relling *et al.*, 1992; Chang *et al.*, 1997). Nonetheless, it is possible that combined enzyme deficiency (*e.g.* relative deficiency in CYP1A2

combined with a glutathione S-transferase deficiency) could further increase an individual's risk for the formation of potentially harmful reactive intermediates. This theory is supported by the analagous finding of Rojas and colleagues (1998) that smokers with combined CYP1A1 and glutathione S-transferase M1 (GSTM1) deficiency showed significantly higher levels of activated covalently bound metabolites than individuals with CYP1A1 or GSTM1 deficiency alone.

Although the *CYP1A2* alleles identified thus far (Section 4.6) would appear not to account for the bi- or trimodality of CYP1A2 activity in a population, it is possible that genetic determinants of variable CYP1A2 activity will be identified. Pre-prescribing genotyping for CYP1A2 activity would then become feasible, and could lead to the judicious use of particularly low doses of clozapine and other drugs that are metabolised by CYP1A2. Furthermore, in combination with assays for polymorphisms in other drug metabolising enzymes such as the glutathione transferases, genotyping for CYP1A2 activity could yield useful information regarding genetic susceptibility to idiosyncratic drug reactions such as agranulocytosis. In the mean time, as it is known that CYP1A2 activity is lower in Orientals and Blacks (Aitchison *et al.*, 2000c), my data suggest that in these groups it would be wise to commence with particularly low doses of clozapine and other CYP1A2-metabolised drugs, and to use a slow dose escalation procedure, monitoring carefully for the emergence of concentration-dependent adverse effects such as sedation and myoclonus.

CHAPTER SIX

DISCUSSION

6.1 Overview of findings and their relevance

In chapter 2, I describe a novel long-PCR assay, developed for the identification of CYP2D6 ultrarapid metabolisers (UMs). This assay was the first assay to be developed for this purpose, has been validated by comparison of results with a subsequently developed assay, and has the potential to provide an estimate of the number of extra copies of the *CYP2D6* coding sequence in UMs.

In Chapter 3, I describe two genotype-phenotype correlation studies, one in French subjects (mainly Caucasians), and one in elderly British Caucasians. The French study showed that it is necessary to genotype for the *CYP2D6*6* allele in French Caucasians in order to predict CYP2D6 PM status with a high degree of accuracy. This had previously been shown for German Caucasians (Sachse *et al.*, 1997), but not for French Caucasians. In the study of elderly British Caucasians, there was one individual with genotype-phenotype discrepancy (**1/*5* genotype, but PM phenotype), which has since been subjected to mutation screening by Dr K Tandon under my supervision. The mean debrisoquine MR (5.67) in the elderly sample is higher than the mean debrisoquine MRs in two previous sets of data on Caucasian volunteers (Daly *et al.*, 1991, mean debrisoquine MR less than 1.0 for N = 73 volunteers; Sachse *et al.*, 1997, mean debrisoquine MR 3.54 for 133 debrisoquine-phenotyped German Caucasians). Moreover, the mean MR for subjects with one functional *CYP2D6* gene was higher

than that in previous studies (Sachse *et al.*, 1997; Leathart *et al.*, 2000). The relatively high overall mean debrisoquine MR may therefore be due to the relatively high mean MR for subjects with *CYP2D6* gene dosage of one; this may in turn was hypothesised to reflect the presence of an intermediate metabolising allele (e.g. *CYP2D6*2*) in the elderly cases with relatively high frequency (*CYP2D6*2* has since been shown not to be in excess, Tandon *et al.*, unpublished data), or reduced CYP2D6 activity in the heterozygous state due to a degree of age-related reduction in CYP2D6 hepatic capacity.

The next section of Chapter 3 reports genetic association studies between CYP2D6 metaboliser status and clinical response to typical antipsychotics including extrapyramidal adverse effects. In a relatively large sample size of treatment-refractory individuals (N = 235), I demonstrated that ultrarapid hydroxylation by CYP2D6 of TAs is not a major cause of failure to respond to treatment with these agents, this being the only study published to date on this topic. A suggestive association between CYP2D6 PM or IM status and a high incidence of adverse effects was shown in a sib pair and a twin pair. A case control study, however, revealed no association between *CYP2D6* gene dosage and intolerance to TAs, or drug-induced parkinsonism, and a trend for a *positive* association (*i.e.* in the opposite direction to that of previous investigators) between the number of functional *CYP2D6* genes and tardive dyskinesia. This study indicated that CYP2D6 PM status or *CYP2D6* gene dosage need not be, in isolation, a risk factor for intolerance to typical antipsychotics (particularly in the case of haloperidol), or specifically DIP or TD. Differences between my findings and those of previous investigators were discussed. It is possible that my finding of a trend for an association with number of functional *CYP2D6* genes and TD, direction of effect the

opposite to that of other investigators, may indicate that there is a real association at this locus with TD, the association being with an element of the locus as yet untested for association with TD (*e.g.* a promoter polymorphism) in linkage disequilibrium with CYP2D6 metaboliser status, or a nearby locus in linkage disequilibrium with *CYP2D6*. This latter explanation would be analogous to the hypothesis proposed by Lerer & Macciardi (2002) to explain the discrepancies in direction of effect (short versus long allele) between the results of different allelic association studies between a serotonin transporter promoter polymorphism (*5-HTTLPR*) and response to antidepressants.

The fact that both the treatment-refractory study and the treatment-intolerant study yielded results in the opposite direction from that predicted may reflect the relatively high proportion of subjects prescribed haloperidol in these studies, and the fact that the precise steps in haloperidol metabolism that CYP2D6 is involved with are at present unclear, but may include the oxidation of reduced haloperidol back to haloperidol, with an inverse correlation between reduced haloperidol levels and clinical response (Bareggi *et al.*, 1990). Moreover, owing to the more potent inhibition of CYP2D6 by phenothiazine antipsychotics as compared to haloperidol (Spina *et al.*, 1991), associations between reduced CYP2D6 activity and antipsychotic adverse effects may be more likely to be found in the case of the phenothiazines than in the case of haloperidol. I suggest that future studies investigating the association between CYP2D6 metaboliser status or gene dosage and adverse effects include the nature and dose of the typical antipsychotic as confounding variables in the analysis.

The next section of Chapter 3 reports a genetic association study of *CYP2D6* and *CYP2C19* genotypes and response to TCAs. Contrary to previous case reports, in this

relatively small sample, a refractory response to treatment with a TCA was not associated with CYP2D6 UM status. A larger prospective study is indicated to further investigate this association, particularly in the case of UMs who have more than 3 functional copies of *CYP2D6*. A significant association was found between the corrected combined TCA level and *CYP2D6* gene dosage ($P = 0.001$), and, controlling for CYP2D6 inhibition, there was a trend for an association between corrected combined TCA level and clinical response to TCA as measured by the percentage change in HDRS ($P = 0.08$). Controlling for CYP2C19 inhibition, there was a significant association between number of functional *CYP2C19* genes and percentage change in HDRS ($P = 0.016$), and between the level of demethylated metabolite and total adverse effect score ($P = 0.01$). There was also a significant association between adverse effect score and CYP2D6 inhibition ($P < 0.005$). CYP2D6 inhibition (by concomitant medication) was significantly associated with both response and adverse effects. The association with CYP2D6 inhibition, and the suggestions of associations between *CYP2C19* gene dosage and clinical response and between demethylated metabolite level and adverse effect ratings warrant replication in a larger prospective study. If replicated, genotyping for *CYP2D6* (including for the *CYP2D6*2* allele) and for *CYP2C19* common variants might assist clinicians in choosing an appropriate dose titration regime and in predicting clinical response to TCAs. This would be consistent with the recommendations of Kirchheiner *et al.* (2001), suggesting dose reductions of approximately 50% for poor metabolisers of CYP2D6 or CYP2C19 in the case of TCAs.

The next section of Chapter 3 reports a trend towards an excess of the *CYP1A2* G_{-3591} allele in those with a poor response to clozapine, but no association with the *CYP1A2*

intron 1 polymorphism. Although no data on cigarette smoking for this sample was available, and an early report (Sachse *et al.*, 1999) indicated an association between the intron 1 C_{-164} SNP and CYP1A2 inducibility by smoking, a more recent report indicates that this polymorphism does not appear to influence the effect of smoking on clozapine levels (van der Weide *et al.*, 2003). It is, however, possible that clozapine itself may induce CYP1A2, and therefore that information on the dose of clozapine and controlling for this variable in the analysis is required. I suggest that any future studies investigating putative associations between *CYP1A2* polymorphisms and response to clozapine should include collection of information on the dose of clozapine, duration of treatment, concomitant medication, and level of smoking, as well as genotyping for other *CYP1A2* polymorphisms. The only factor that emerged as significantly associated with response was gender, consistent with the higher CYP1A2 activity in males as compared to females. The mechanism of the effect of oestrogens on CYP1A2 is not well understood, but by analogy with the effects of other steroids on regulation of gene expression, may be exerted by a transcriptional effect on the *CYP1A2* promoter. It is then possible that a *CYP1A2* promoter variant that is associated with the inhibition of CYP1A2 by a variety of agents including oestrogens might be associated with clozapine response. The $T_{-3591}G$ SNP might be in linkage disequilibrium with such a variant. The trinucleotide repeat region that I have identified, $(AAC)_6$ at –4382 to –4399, warrants investigation as a candidate for functional variation, and further mutation screening of the *CYP1A2* promoter, including the XREs and regions that are conserved across species, and could be involved in the transcriptional effect of oestrogens, could yield interesting data.

Chapter 4 describes mutation screening of part of the *CYP1A2* 5' flanking region, and the identification of a $T_{-3591}G$ substitution, a $G_{-3595}T$ substitution, and a T_{-3605} insertion (Aitchison *et al.*, 2000a). Genotyping revealed a significantly higher frequency of the G_{-3591} allele in Taiwanese as compared to Caucasians and African Americans. Functional characterisation of the G_{-3591} and T_{-3595} variants, and the combination of the two, was undertaken, which revealed that the T_{-3595} variant was associated with a slightly higher constitutive *CYP1A2* promoter activity. However, this was only by a factor of 1.2, which is unlikely to be of significance *in vivo*. There did not seem to be any functional consequence in terms of TCDD-induced activity for the mutations tested. Therefore, although novel variants in the 5' flanking region were identified, they did not appear to have a functional consequence.

Genome walking with sequencing, and sequencing of a BAC clone revealed sequence discrepancies compared with the sequence published by Quattrochi & Tukey (1989) and my 532 bp novel 5' flanking *CYP1A2* sequence. Comparison versus GENBANK sequence AF253322 revealed the presence of a trinucleotide repeat, $(AAC)_6$, and a poly-T and a poly-A sequence in my sequence versus the GENBANK sequence, and three possible SNPs. These may represent polymorphic sites, or, alternatively, sequencing errors in the GENBANK sequence owing to the method employed ("shotgun cloning"), and the likely instability of the microsatellite and poly-T and poly-A sequences. Further sequence analysis across this region is required to confirm these potentially polymorphic sites, followed by functional studies as indicated.

Chapter 5 describes a study of clozapine pharmacokinetics and pharmacodynamics in CYP1A2-null mice, which showed that CYP1A2 contributes significantly to the demethylation of clozapine *in vivo*, with CYP1A2 being the major determinant of

clozapine clearance in wild-type mice. After clozapine administration, the CYP1A2-null mice were significantly more drowsy, showed more motor impairment, and had more myoclonus than the wild-type mice. This suggests that in patient groups in which CYP1A2 is relatively low in activity (Orientals, Blacks, female non-smokers) that it would be wise to use a slow dose titration procedure for clozapine and other CYP1A2-metabolised drugs, monitoring carefully for the emergence of concentration-dependent adverse effects.

6.2 Methodological issues in pharmacogenetics research

Much of the world-wide research effort in pharmacogenetics has so far been disappointing, and in this respect, the results of some of my studies (*e.g.* investigating the number of functional *CYP2D6* genes and response to typical antipsychotics) are not unusual. There are a number of methodological issues that may have contributed to this, as has been discussed by various authors (Hodge 1994; Elston 1998; Malhotra and Goldman 1999; Paterson *et al.*, 1999; Veenstra-VanderWeele *et al.*, 2000), and include heterogeneity in clinical, genetic, and statistical methodology employed by different research groups, and the complexity of the phenotype of drug response. All of these issues are well illustrated by studies on the pharmacogenetics of extrapyramidal side effects (EPS) including tardive dyskinesia (Lerer *et al.*, 2002), and also by studies on the pharmacogenetics of clozapine response (discussed in Aitchison & Gill, 2002).

6.2.1 Heterogeneity in clinical methodology

Heterogeneity in clinical methodology between different studies refers to the variation in diagnostic criteria employed, clinical scales used, length of washout periods, *etc.* For example, Vandel *et al.* (1999) and Chou *et al.* (2000), and my studies on TA

adverse effects including DIP and TD differed in that the samples of Vandel *et al.* and Chou *et al.* were mixed in terms of diagnosis and drug (including non-psychotic illnesses and *e.g.* antidepressants as well as antipsychotics), and the clinical ratings (EPS assessment using the Simpson-Angus scale plus clinical examination and interview to diagnose dystonia or akathisia according to the Leo (1996) criteria for Vandel *et al.*, Simpson Angus Scale for Chou *et al.*, Webster's Rating Scale for Parkinsonism and the AIMS for TD for my sample). The mixed nature of the samples might well be relevant to the difference in findings, as the association between adverse drug events and *CYP2D6* gene dosage may be stronger in the case of antidepressants than in the case of antipsychotics (Chen *et al.*, 1996). Similarly, Armstrong *et al.* (1997) and Scordo *et al.* (2000) studied a variety of movement disorders, including parkinsonism and dyskinesia in the case of the former, and dystonia, parkinsonism, and TD in the latter, whereas I separated out DIP and TD cases for the purpose of the analysis. There is also the issue of whether or not the diagnosis of TD was made by more than one assessment; two sequential clinical assessments, at least three months apart are necessary to fulfil the Schooler & Kane (1982) criteria for persistent TD, and few studies (*e.g.* Kapitany *et al.*, 1998; Andreassen *et al.*, 1997) have complied with this, most assessing at one time point only, which can give only a diagnosis of RDC probable TD. Finally, there is the issue of heterogeneity in terms of which antipsychotics were prescribed, which could in fact be the most important factor in accounting for discrepancies in findings (sections 3.2.3.3 and 3.2.4.2.3).

6.2.2 Heterogeneity in genetic methodology

Examples of heterogeneity in genetic methodology include the use of phenotyping (*e.g.* Arthur *et al.*, 1995) as well as genotyping in the analysis, and the *CYP2D6* alleles

investigated (*CYP2D6*3-5* and gene amplification by RFLP analysis in Arthur *et al.*, (1995); *CYP2D6*3-5* in Armstrong *et al.* (1997); *CYP2D6*3-7* in that of Andreassen *et al.* (1997); *CYP2D6*3-5* in that of Kapitany *et al.* (1998); *CYP2D6*3-4*, and **10* in Ohmori *et al.*, 1998; wild-type, reduced activity, and non-functional alleles including *CYP2D6*2-6* and **9-10* in Vandel *et al.* (1999); *CYP2D6*10* only in Lam *et al.* (2001); and *CYP2D6*2* in Ohmori *et al.* (1999); *CYP2D6*3-6* and gene amplification in Scordo *et al.* (2000); *CYP2D6*3-5* and gene amplification in my study). My study and those of Arthur *et al.* (1995) and Scordo *et al.* (2000) are the only studies to date to have included assaying for *CYP2D6* gene amplification events. Of note, in Scordo *et al.*'s study, no difference in the distribution of homozygous EM, heterozygous EM, and UM were found between those with and without EPS. Although in their study all 4 (out of a total N of 119) PMs had a history of EPS, they acknowledged that concluding that there was an association between PM status and EPS was premature, owing to the small number of PMs. Of note, the 3 PMs in my study (N = 72) had neither DIP nor TD. The apparent association between PM status and EPS in Scordo *et al.* (2000) study and the absence of such an association in my study could then have occurred by chance, owing to the small numbers of PMs in both studies.

6.2.3 Heterogeneity in statistical methodology

Heterogeneity in statistical methodology refers to, for example, whether a parametric (using *e.g.* total AIMS score) or categorical analysis (*e.g.* using presence or absence of TD, defined as probable or persistent) is conducted, and whether or not effort is made to control for confounding variables. Most groups (Andreassen *et al.*, 1997; Kapitany *et al.*, 1998; Ohmori *et al.*, 1998 and 1999; Lam *et al.*, 2001; my study) have conducted a categorical analysis (mostly using RDC probable TD); some (Arthur *et al.*, 1995;

Ohmori *et al.*, 1998 and 1999; my study) have employed parametric analysis using total AIMS score, or, in the case of Kapitany *et al.* (1998), total Tardive Dyskinesia Rating Scale score; others (my study and Ohmori *et al.*, 1998) have additionally used an AIMS score of at least 6, or mean AIMS score in different genotyping categories (Andreassen *et al.*, 1997; Ohmori *et al.*, 1998 and 1999; Scordo *et al.*, 2000), or AIMS score of at least 4 (Armstrong *et al.*, 1997). For those studies that have included an analysis by AIMS score, three have been negative (Arthur *et al.*, 1995; Ohmori *et al.*, 1999; my study), and only one positive (Ohmori *et al.*, 1998). As to controlling for confounding variables, this was performed using logistic regression and/or linear regression analysis by Ohmori *et al.* (1998, 1999), and my study, but not by other investigators. These differences in statistical approach may have contributed to the different conclusions reached (see Section 3.2.4.2.3.).

A further statistical issue is the question of power: the power of my sample to test the hypothesis by logistic regression was 0.999 for the 72 subjects rated for probable TD (using values from the data of: affected proportion 0.18, odds ratio 4.86, $R^2 = 0.0469$, $\alpha = 0.05$), and 0.96 for the 66 subjects rated for persistent TD (affected proportion = 0.17, odds ratio = 3.49, $R^2 = 0.0348$, $\alpha = 0.05$; calculations performed using NCSS 2000). Comparable sample sizes were used by Andreassen *et al.* (1997; N = 100), Ohmori *et al.* (1998, 1999; N = 100), and Scordo *et al.* (2000; N = 119, 15 affected with TD), but relatively small samples were used by Arthur *et al.* (1995; N = 16 affected with tardive dyskinesia only), Kapitany *et al.* (N = 45), and Lam *et al.* (N = 38 affected with TD, 38 without TD). It would appear that none of the samples studied to date had sufficient power to adequately address the question of the possible contribution of CYP2D6

variants to the genesis of EPS including TD, especially if consideration is also to be made regarding a possible interactive effect of CYP2D6 with other loci.

6.2.4 The complexity of the phenotype

Apart from heterogeneity in methodology in the studies of the pharmacogenetics of EPS including TD, the inconsistent nature of the findings may well be related to the complex nature of the phenotypes being addressed. Subtypes of EPS include DIP, dystonia, akathisia, and TD, and different groups have included different subtypes as discussed above. Furthermore, within subtypes, there may be subdivisions; for example, for TD, risk factors include high lifetime antipsychotic exposure, presence of a movement disorder, negative symptoms, cognitive impairment, diabetes, alcohol or drug abuse, female gender, smoking, age (in most studies), and, in some studies, affective disorder (Kane & Smith, 1982; Kane, 1992; Yassa & Jeste, 1992; Wolf *et al.*, 1983; Kane *et al.*, 1986; Woerner *et al.*, 1991; Caliguiri *et al.*, 1991; Waddington 1995a; van Os *et al.*, 1997; Muscettola *et al.*, 1993 and 1999; Sections 3.2.1 and 3.2.4.2.3). Different candidate genes can be postulated to be contributing to different subtypes of TD, *i.e.* TD may be genetically heterogeneous. Thus, for example, it may well be that the TD associated with movement disorder, cognitive impairment, and negative symptoms should be subjected to a separate pharmacogenetic analysis from that associated with, for example, smoking. Polymorphisms in different candidate genes may interact, each contributing a small proportion to the total variance observed in the phenotype (*i.e.* polygenic inheritance), which may lead to false negative findings (type I errors). Furthermore, Quinn *et al.* (2001) report that the prevalence of involuntary movements approaches 100% over a lifetime trajectory for individuals with schizophrenia chronically medicated with TAs. The point at which one samples

subjects for a pharmacogenetic association study of TD then becomes crucial: too early and not enough individuals may have passed through the age of association of risk with the particular genetic risk factor(s) being investigated, and too late and the number of affected individuals becomes so great that association with any putative genetic risk factors is masked.

6.3 The pharmacogenetics of the future: pharmacogenomics

With the advent of the "new genetics," the term pharmacogenomics has arisen, which has been variously defined (Housman & Ledley, 1998; Persidis, 1998; Regalado, 1999; Rioux, 2000; Masellis *et al.*, 2000; Aitchison & Gill, 2002), but encompasses the application of genomics to the study of pharmacogenetics, or pharmacogenetics in a postgenomic era (*i.e.* after the availability of the draft sequence of the human genome). Although the two terms are often used interchangeably, it may be useful to try and retain the distinction between the two.

Pharmacogenetics is the study of the relationship between definable genetic factors (polymorphisms or mutations) and clinical drug response endophenotypes, with the aim of generating information that may lead to rational drug prescribing, based on individual genetic profile. Pharmacogenomics may be considered a branch of functional genomics. The availability of the draft sequence of the human genome and recent advances in molecular genetics means that there is now an unprecedented opportunity to study all genes in the human genome, including genes encoding drug metabolising enzymes, drug targets, regulatory mechanisms, and post-receptor messenger machinery in relation to drug safety and efficacy (Özdemir, Shear, & Kalow, 2001). Pharmacogenomics includes the utilisation of techniques such as high

throughput genotyping of genome-wide markers in order to identify genomic "hotspots" corresponding to susceptibility loci contributing to response or adverse effect profiles by linkage and/or association. It also includes bioinformatic analysis of the wealth of sequence data now available, in order to identify novel sequence variants in or near candidate genes, followed by experimental confirmation of such variants, with functional characterisation (for example of variants in promoter sequences, 5' or 3' untranslated regions, or intronic sequence and their impact on levels of gene expression / mRNA splicing). More recently, it has been shown that gene expression microarrays can be used to correlate patterns of gene expression (upregulation or downregulation) with drug effect or toxicity – this has been termed "transcriptome profiling," and has been employed with some promising results (Alen, 2001).

The short-term goal of pharmacogenomics is the same as pharmacogenetics, that is, to elucidate how individual genetic make-up influences the response to or side-effects of a particular drug (Masellis *et al.*, 2000). A methodological difference between the two fields is that pharmacogenetics is hypothesis driven, requiring *a priori* knowledge of drug pharmacology, whereas (e.g. in the search for genomic "hotspots"), pharmacogenomics may not be. The long-term vision of pharmacogenomics is that the discovery of genetic influences on drug action will lead to the development of new diagnostic procedures and therapeutic products that enable drugs to be prescribed selectively to patients for whom they will be effective and safe (Housman & Ledley, 1998). In the US, in 1994, adverse drug reactions (ADRs) were between the fourth and sixth leading cause of death amongst hospitalised patients (Lazarou *et al.*, 1998). The expectation of pharmacogenomics is that subgroups of patient populations might be identifiable - those who are likely to respond better to a given drug than the rest of

the population, and those who are less likely to have an ADR - and that drugs will then be able to be selectively marketed to those patient groups (population segmentation or stratification by genetic profile). This could lead to significant cost savings, both in the cost of drug development, and costs to the individual and to the health care provider. Clinical trial design might become more efficient and drugs might reach the market faster, and stay there longer (Spallone & Wilkie, 1999). If a patient could be identified as being unlikely to respond well to a given drug, then the cost of months of ineffective treatment – costs both personal and economic – could be avoided. Similarly, those at high risk of an ADR for a given drug could be saved resulting morbidity or even mortality. Pharmaceutical companies might even resurrect drugs that have been taken off the market, if subgroups of patients who were at risk of the particular offending ADR could be identified and excluded from the prescribing license. For example, consider the ADR of agranulocytosis on clozapine treatment (section 5.4), which has the consequence of prescription in the USA and UK under a restricted license, with strict haematological monitoring. If the subgroup of patients likely to be at high risk of agranulocytosis could be identified, then this patient group could be excluded from the prescribing license, with the result that haematological monitoring might not be required for the rest of the population prescribed clozapine, or be required at a lower monitoring frequency. This, of course, would require a test for susceptibility to agranulocytosis with a high level of sensitivity and specificity.

6.4 Suggestions for future directions in pharmacogenetics/genomics

6.4.1 Study design

From consideration of the methodological issues, a number of recommendations may be made regarding future pharmacogenetics and pharmacogenomics studies. Firstly, the design of the clinical data set is crucial. Prospective studies that are specifically designed for pharmacogenetic or pharmacogenomic analysis and include the power and rigor of design necessary to test pharmacogenetic hypotheses are most likely to yield informative data (Pickar and Rubinow, 2001; Masellis *et al.*, 2000). Ideally, standardised diagnostic interviews should be used, a clearly defined washout period should be employed, with prior treatment response and baseline symptom level recordings, and prospective recordings of clinical response and adverse effects using standardised rating instruments should be made at predefined, theoretically justifiable time points. In addition, the drug dose and plasma drug levels (both serially, at different response times) should be determined, and details of standard demographic variables (age, sex, and ethnicity), other clinical variables (e.g. age at onset of diagnosis, comorbid diagnosis, family history, symptom complex), and any others pertinent to the psychotropic drug being evaluated should be determined. Exclusion criteria should be defined *a priori*, as should the criteria for clinical response (e.g. mean change in clinical score over time, controlling for baseline, Basile *et al.*, 1999).

6.4.2 Statistical issues

Secondly, some recommendations regarding statistical issues may be made. In the case of pharmaco*genetic* studies, calculations of sample size required for adequate power should also be conducted *a priori*, taking into consideration allele frequencies for the specific polymorphisms to be examined, and the number of candidate genes to be tested (Masellis *et al.*, 2000). This may not, however, be possible for pharmaco*genomics* studies, in which e.g. marker allele frequencies (particularly in disease populations)

may not be known. Similarly, it may be appropriate to define *a priori* an appropriate significance level given the number of candidate loci and hypotheses to be tested. Finally, in the data analysis, the relative effect size contributed by each candidate gene polymorphism should be estimated (Masellis *et al.*, 2000).

In order to control for population stratification, methods such as the Transmission Disequilibrium Test (TDT; Spielman & Ewens, 1996), or "Genomic Control" (GC; Bacanu *et al.*, 2000), or the method of Pritchard and colleagues (Pritchard & Rosenberg, 1999; Pritchard *et al.*, 2000) may be used. The TDT has been extended for use with quantitative traits (and response to drug treatment measured on a linear scale may be viewed as a quantitative trait), is resistant to confounding (Allison, 1997; Rabinowitz *et al.*, 1997), and has particularly high power if the most extreme 20% of the phenotypic distribution is selectively sampled. However, adequate sample sizes may be difficult to attain, given that it can be difficult or impossible to collect clinical information and DNA from parents of relatively old affected individuals, and also, with TDT, only heterozygous parents are informative. Whenever parents are homozygous, the genotyping data are effectively useless, with consequent loss of study power. An alternative TDT approach to the standard parent-child trio uses samples of sibling pairs to test for association (Spielman & Ewens, 1998).

The analysis may also be improved by genotyping for a number of different polymorphic sites at loci of interest, so that haplotypes may be constructed. Some high throughput genotyping strategies (especially those involving pooling) may not automatically yield haplotype data, but using haplotype estimation maximization algorithms (Long *et al.*, 1995), it is possible to assign haplotypes. Usually haplotype

analysis provides the benefit of more information about the likelihood that a particular gene is associated with the phenotype of interest, and has been performed with success (e.g. haplotype analysis of the β2-adrenergic receptor gene and response to bronchodilators, Drysdale *et al.*, 2000). The power and validity of haplotype-based analysis is increased by the grouping of haplotypes according to their most likely common origin (Templeton *et al.*, 1987). A detailed haplotype analysis of the exon 3 VNTR of the *DRD4* (dopamine D4 receptor) gene has been provided (Ding *et al.*, 2002).

"Genomic Control" refers to the use of 20-60 SNPs spread throughout the genome (Devlin & Roeder, 1999); Bacanu *et al.*, 2000), in order to generate an estimate of the degree of population stratification in an apparently homogeneous population. The method of Pritchard and colleagues (Pritchard & Rosenberg, 1999; Pritchard *et al.*, 2000) is similar, but uses 15-20 unlinked microsatellite markers, in a method that has greater power to detect stratification than biallelic markers such as SNPs.

6.4.3 Candidate pathway analysis

Thirdly, there are recommendations to be made in regard to the candidate genes chosen for analysis. Both pharmacokinetic and pharmacodynamic genetic factors should be investigated, as well as genes that appear to be of aetiological significance in the relevant disease process. An illustration of this is candidate pathway analysis (Craig & McClay, 2002), in which, for example for a pharmacogenetic analysis of response to proserotonergic antidepressants, one might include the genes involved in serotonin synthesis and breakdown, in addition to the serotonin transporter and regulators of serotonin transporter response (e.g. the presynaptic $5HT_{1A}$-receptor, Smeraldi *et al.*,

1998; Kelsoe *et al.*, 1998). This suggestion, though, may lead to the inclusion of a large number of candidate genes (e.g. BDNF, Stahl, 2000; third messenger systems such as kinases; other neurotransmitter systems functionally connected to the serotonergic system, Schafer, 1999; etc.), and a balance may have to be struck between what is desirable to investigate and what is practical, given the sample size and power considerations. If possible, a hierarchy of which genes are presumed to be "better candidates" than others should be constructed, using the existing literature, which will not be easy unless pilot pharmacogenetic studies have been conducted.

6.4.4 Open system approaches

Some of the most interesting results to date (reviewed in Chen *et al.*, 2001; Wang *et al.*, 2001; Rohlff, 2001) in the field of pharmacogenomics have come from the use of open system approaches, and it may be that significant effort should be expended in the employment of these. "Open" system approaches are "open" rather than "closed" in that they are not hypothesis-driven, and hence have greater potential for truly novel discoveries regarding the mechanisms of psychotropic drug action. Such open system approaches include genome-wide scans, differential gene expression studies, and proteomic approaches.

6.4.4.1 Genome-wide scans

In pharmacogenomic genome-wide scans, all potential genes are evaluated for association with a psychotropic phenotype, using markers spaced throughout the genome. These markers may be microsatellite markers (historically used *e.g.* for linkage analysis, and previously at a spacing that gave incomplete coverage of the genome), or single nucleotide polymorphisms (SNPs). SNPs are the most common

genetic variant in man (Schork *et al.*, 2000), occurring at a density of approximately 1 per 1000 bp when comparing two unrelated individuals; a genome-wide map of SNPs has been published (International SNP Map Working Group, 2001), and is continuously being updated. It has been estimated that approximately 30,000 SNPs are sufficient to undertake a genome-wide scan (Collins *et al.*, 1999); however, this view has been questioned (Weiss & Terwilliger, 2000). The density of SNP markers required depends on the average degree of linkage disequilibrium extending from each SNP. The work of Reich *et al.* (2001) suggested that linkage disequilibrium extended 60 kb from common alleles in a sample of subjects of Northern European descent in the United States, and was similar in a Swedish sample, but a much smaller degree of linkage disequilibrium (LD) was found in a Nigerian sample. This paper has, however, been criticised by Weiss & Clark (2002), who commented that the LD estimate for the Nigerian sample was inaccurate due to inequality in sample size between this and the Caucasian samples, degree of inbreeding, and method of SNP ascertainment.

6.4.4.2 Differential gene expression studies

In open differential gene expression studies, all the genes expressed in a system such as a cell or a specific brain region (transcriptome) may be profiled. Two methods have been used to date for such studies: differential display polymerase chain reaction (DD-PCR), and expression microarrays. Most of the studies to date have been conducted in animals.

DD-PCR provides a comprehensive approach to identifying genes that are differentially expressed at the mRNA level, especially when relatively small changes in gene expression are expected. In this technique, total RNA is reverse-transcribed to cDNA

using an 'anchored' oligo(dT) primer (Liang *et al.*, 1994). The cDNA is then amplified and radiolabelled in a series of reactions, each using one 3' anchored primer and an arbitrary oligonucleotide as the second primer. The use of an arbitrary, rather than specific primer, results in the amplification of as many different mRNA species as possible. Matching reactions (*i.e.* generated with the same set of primers for all experimental RNA samples, in duplicate) are then run side-by-side on a denaturing gel, and the bands analysed. Bands exhibiting different intensities between experimental treatment groups (*e.g.* samples treated with a specific psychotropic agent versus untreated) represent candidate transcripts with altered expression levels, which are then subjected to further analysis. Findings are then validated by performing *e.g.* real-time PCR and *in situ* hybridisation, and further integrative studies may be conducted in order to establish the relationship between the molecular/cellular changes and facets of the therapeutic response being studied (Ikonomov & Manji, 1999).

DD-PCR has been used by several laboratories to study the effects of mood stabilisers (such as lithium and valproate) on gene expression (Chen *et al.* 1999a, 1999b, 2001; Hua *et al.*, 2000; Wang *et al.*, 1996, 1999a, 1999b), which has led to interesting results, including the identification of a novel lithium-regulated gene (Wang *et al.*, 1999b) and unexpected targets of the long-term actions of mood stabiliser genes, such as the cytoprotective protein human B-cell lymphoma protein-2 (bcl-2; Chen *et al.*, 1999b).

There are now available expression microarrays (Affymetrix, Santa Clara, CA, GeneChip^{R} Human Genome U133 set), that cover most of the human transcriptome (over 10^{6} oligonucleotides, covering more than 39,000 transcript variants, representing more than 33,000 human genes). These high density microarrays, with at least 300,000

polydeoxynucleotides per array (Lipshutz *et al.*, 1999), allow the analysis of large numbers of human expressed sequence tags (ESTs), each EST being represented by 16-20 pairs of specific 25-mer oligonucleotides (each probe pair containing a perfect match and a mismatched oligonucleotide). Total RNA is reverse-transcribed into cDNA, then transcribed into cRNA in the presence of fluorescent label, which is hybridised to two identical microarrays, and the signal patterns analysed (Strakhova & Skolnick, 2001). The employment of multiple probe pairs is to improve the specificity of the experiment, more than half of the perfect-match probes having to produce a hybridisation signal in order for the hybridisation to a particular gene sequence to be considered real. The presence of mismatched oligonucleotides facilitates efficient subtraction of non-specific hybridisation and background signal. It is generally accepted that the GeneChip arrays are able to detect changes in mRNA expression of at least 2-fold (Strakhova & Skolnick, 2001), although it is possible that sensitivity may extend down to 1.4-fold with modification of the method of analysis (J DeBellaroche, presentation at Davos, February 2002). Therefore, while microarray technology presents an attractive alternative to labour- and time-consuming DD techniques, its application to finding unknown sequences and modest changes in gene expression levels (1.5-fold or less) is likely to be problematic (Strakhova & Skolnick, 2001).

Nonetheless, if used as a complement to DD, it may reveal genes that are coregulated with those identified by DD. Indeed, this technique has been successfully used to show that genes coding for a particular system or pathway (e.g. a neurotransmitter pathway) are differentially expressed in concert (Hakak *et al.*, 2001; Mirnics *et al.*, 2000). In the field of neuropharmacology, it has identified genes differentially expressed in the rat brain following treatment with the antidepressant sertraline (Yamada *et al.*, 2000), and

on exposure to Δ^9-tetrahydrocannabinol (the primary psychoactive agent in cannabis) (Kittler *et al.*, 2000). There is evidence that the brain is the organ that exhibits the greatest degree of complexity of gene expression (Colantuoni *et al.*, 2000), and hence pharmacogenomics studies using differential gene expression methodologies hold great promise.

6.4.4.3 Proteomics and neuropsychopharmacology

A review of the application of proteomics to the study of neuropsychiatric disorders has been provided recently (Rolff, 2001). Proteomics describes techniques that aim to profile all proteins expressed in a particular system (*e.g.* a cell, body fluid, or tissue). The gene expression profile in terms of transcripts may not equate to the proteomic profile because gene transcription and protein expression are often not unambiguously linked and may be regulated separately (Guygi *et al.*, 1999), including *e.g.* post-translational protein modifications, and also, in the case of postmortem samples, postmortem delay in human brain tissue affects mRNA more than protein (Edgar *et al.*, 1999). Current proteomic approaches include 2-dimensional gel electrophoresis, mass spectrometry, and protein chip arrays (Senior, 1999; Parekh & Lyall, 2000). In neuropsychopharmacology, these may be applied to the study of cerebrospinal fluid (CSF) proteins (Sharma *et al.*, 1997; Thompson *et al.*, 1999), plasma (Müller *et al.*, 1997), and CNS tissues (Davidsson *et al.*, 1999; Greber *et al.*, 1999; Johnston-Wilson *et al.*, 2000). However, to date most of these applications have been studies related to neuropsychiatric disease aetiology rather than to psychotropic drug response. Proteomics is at present under-explored in neuropsychopharmacology, but is predicted to lead to more selective and efficacious treatments (Lieberman, 2000), including the

development of both novel therapeutics and second-generation compounds derived from an improved understanding of the mechanisms of action of existing therapies.

6.5 Projects arising from work conducted in this thesis

Mutation screening of CYP2D6 by DHPLC in samples with genotype-phenotype discrepancy from my studies is being undertaken. So far this has revealed two subjects with an Ile109Val amino acid substitution, which would appear to be associated with reduced CYP2D6 activity (Tandon *et al.*, 2002a).

I have extended my study on the pharmacogenetics of antidepressant response to include subjects on other antidepressants, and pharmacodynamic candidate genes, as well as drug metabolising enzymes. I am also involved in a proposal for funding under the European Framework 6 Programme (FP6), which proposes to use functional genomics and proteomics in order to identify genomic loci and their functional correlates corresponding to clinical response to antidepressants.

A large sample (N>250) individuals chronically treated with typical antipsychotics, who have been rated for TD using the AIMS (Abnormal Involuntary Movements Scale) according to the Schooler and Kane criteria (1982), is being amassed through collaborations. Using this sample, Dr Tsapakis, under my supervision, will be extending the pharmacogenetic association study of tardive dyskinesia to include other relevant pharmacodynamic and drug metabolising candidates.

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Failure to respond to treatment with typical antipsychotics is not associated with CYP2D6 ultrarapid hydroxylation

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Aims To investigate whether or not there is a correlation between failure to respond to typical antipsychotics and CYP2D6 ultrarapid metaboliser status.

Methods CYP2D6 phenotype (metaboliser status) was assigned following genotyping for gene duplication, as well as for the *CYP2D6*3*, *CYP2D6*4*, and *CYP2D6*5* null alleles in 235 treatment-refractory patients and 73 nonrefractory patients.

Results Four (1.7%) of the 235 treatment-refractory subjects were positive on the duplication assay, but, of these, two were found to represent duplications of a null allele (*CYP2D6*4*), therefore leaving only two (0.85%) positive for duplication of a wild type allele (ultrarapid metabolisers). Three (4.1%) of the nonrefractory subjects had a genotype consistent with ultrarapid metaboliser status. Fisher's exact test gave a two-tailed *P* value of 0.091, i.e. a trend towards an excess of ultrarapid metabolisers in the nonrefractory group, which was in the opposite direction to that predicted by our hypothesis.

Conclusions Although the results show a trend towards an excess of ultrarapid metabolisers in the nonrefractory group, the percentages in the two groups of patients are both within the range for ultrarapid metabolisers in Caucasian populations. Our data are not consistent with ultrarapid metaboliser status being a major cause of failure to respond to typical antipsychotics.

Keywords: CYP2D6, genotype, metabolism, pharmacokinetics, treatment-refractory, typical antipsychotics

Introduction

The cytochrome P450 enzyme CYP2D6 is a polymorphic enzyme which contributes significantly to the pharmacokinetics of most typical antipsychotics [1]. Between 0.5% and 7% of Caucasians have very high enzyme activity [2, 3] and are known as ultrarapid metabolisers, or UMs. This high enzyme activity is due to the presence of 2 or more copies of a functional *CYP2D6* allele existing in tandem on the same chromosome [2, 4–6]. At the other end of the metabolic spectrum are individuals who are homozygous for null alleles (poor metabolisers or PMs), representing about 7–9% of most Caucasian populations. The remainder of the population (excluding PMs and UMs) are known as extensive metabolisers (or EMs).

Two patients with ultrarapid metaboliser status have been described for whom tricyclic antidepressants at doses beyond the usual therapeutic range were required in order to achieve a therapeutic response [4]. Up to 30% of patients with schizophrenia who are prescribed typical antipsychotics are treatment-resistant [7]. The term treatment-resistant includes those who are treatment-refractory (show inadequate clinical response) and those who are treatment-intolerant (exhibit adverse responses). We hypothesized that patients with schizophrenia who were refractory to treatment with typical antipsychotics would be more likely to be ultrarapid metabolisers, as compared with patients who responded to typical antipsychotics. If the hypothesis were confirmed, it could form the rationale for a preprescribing genotyping assay to predict patients who would be less likely to respond well to typical antipsychotics at standard doses, and therefore assist the process of clinical dose finding and/or the more rapid progression of such patients on to an atpyical antipsychotic not subject to the CYP2D6 polymorphism.

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Methods

Our 235 treatment-refractory subjects came from a sample of 246 patients treated with clozapine in the United Kingdom (UK). All of these patients were resistant to treatment with typical antipsychotics, and had a diagnosis of schizophrenia or schizoaffective disorder (clozapine prescribing restrictions in the UK, UK Clozaril Patient Monitoring Service). Prescribing consultants provided data regarding whether their patients were refractory to typical antipsychotics, intolerant of typical antipsychotics, or both refractory and intolerant of typical antipsychotics. Out of the 246 patients on clozapine there were a total of 235 who were refractory, or refractory *and* intolerant to typical neuroleptics. The standard definition of treatment-refractory schizophrenia has been provided by Kane *et al.* [7]: (1) at least three periods of treatment in the preceding 5 years with antipsychotics (from at least two different chemical classes) at doses equivalent to or greater than 1000 mg day^{-1} of chlorpromazine for a period of 6 weeks, each without significant symptomatic relief, and (2) no period of good functioning within the preceding 5 years. Data regarding which typical antipsychotics had been prescribed were not available. However, the most commonly prescribed typical antipsychotic prior to switching to clozapine in the UK for the period during which our sample was collected was haloperidol (Novartis, personal communication).

Our comparison group, nonrefractory to typical antipsychotics, comprised 73 patients from the Maudsley and Bethlem Royal Hospitals NHS Trust. Sixty-six of these had been treated with various antipsychotics, mostly haloperidol (approximately 60%) or fluphenazine, at doses equivalent to at least 100 mg chlorpromazine daily for at least 12 months prior to assessment and had DSM-IIIR schizophrenia. Information regarding duration of treatment was not available for the remaining seven subjects, but six had been treated with the equivalent of at least 100 mg chlorpromazine daily, while the remaining patient received 15 mg flupenthixol decanoate by depot injection fortnightly, the equivalent of 75 mg chlorpromazine daily. Six of these seven subjects had a clinical diagnosis of schizophrenia; one had a diagnosis of affective psychosis. As the patients were treated with a variety of antipsychotics, we converted all the prescriptions to chlorpromazine equivalents according to British National Formulary guidelines, in order to assess whether or not there was a relationship between the magnitude of the dose and the CYP2D6 genotype.

Ethics Committee approval was obtained for the study on all subjects, and, as there were insufficient numbers of non-Caucasians in the sample for the analysis to be informative, all non-Caucasians were excluded.

DNA was extracted from blood collected in EDTA tubes using the Nucleon II kit (Nucleon Biosciences, UK). *CYP2D6* gene duplication was detected by the long-PCR method of Løvlie and colleagues [8], using the Expand Long Template PCR System (Boehringer Mannheim, UK), and primers 5′-TCCCCCACTGAC CCAACTCT-3′ and 5′-CACGTGCAGGGCAC CTAGAT-3′. The PCR was performed in a final volume of 25 μl including 2.5 μl Boehringer buffer 1, 4.5 μl of a 2 mM solution of each dNTP, 0.5 μl of each primer (10 μM solutions), and 0.25 μl of Boehringer enzyme mix (Taq/Pwo). Boehringer buffer 1 contains 20 mM Tris-HCl pH 7.5, 100 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA, 0.5% (v/v) Tween$_R$ 20, 0.5% (v/v) Nonidet$_R$ P40, 50% glycerol (v/v), and 1.75 mM $MgCl_2$. Cycling conditions were modified as follows: initial denaturation for 2 min at 94° C, 35 cycles of 93° C for 10 s, 60° C for 30 s and 68° C for 5 min, followed by an elongation step of 68° C for 7 min.

As cases have been described in which there are extra copies of a nonfunctional or null *CYP2D6* allele [8–10], it is necessary to assay for nonfunctional *CYP2D6* alleles as well as for the presence of a duplication event in order to confirm ultrarapid metaboliser status. We therefore assayed for the *CYP2D6*3*, *CYP2D6*4*, and *CYP2D6*5* null alleles. *CYP2D6*4* and *CYP2D6*5* are the most common and next most common null alleles, respectively; analysis for *CYP2D6*4*, *CYP2D6*5*, and *CYP2D6*3* should detect 90–95% of null alleles in a European Caucasian population [11–13].

The *CYP2D6*3* and *CYP2D6*4* point mutation alleles were detected by PCR followed by restriction enzyme digestion as in the method of Smith *et al.* [14], with minor modifications. For the *CYP2D6*3* assay, primers 5′-ATGAGCTGCTAACTGAGCCC-3′ and 5′-CCGA GAGCATACTCGGGAC-3′ were used in a total reaction volume of 25 μl containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.001% (w/v) gelatin, 3 mM $MgCl_2$, 0.2 mM each dNTP, 0.25 μM each primer, and 1.25 U AmpliTaq (Perkin-Elmer, UK). Cycling conditions were: initial denaturation at 94 °C for 3 min, 30 cycles at 95° C for 1 min, 60° C for 30 s, and 72° C for 1 min, followed by final elongation at 72° C for 10 min. PCR products were digested using *Hpa*II, and analysed on a 3% agarose gel, together with a 1kb ladder (Gibco BRL). For the *CYP2D6*4* assay, we used primers 5′-GCCTTCGCCAACCACTCCG-3′ and 5′-AAATCCTGCTCTTCCGAGGC-3′ and the same conditions as for *CYP2D6*3*, except a $MgCl_2$ concentration of 1.5 mM. PCR products were digested with *Bst*NI and analysed on a 3% agarose gel as above.

The *CYP2D6*5* gene deletion allele was assayed by long-PCR using the GeneAmp XL PCR kit (Perkin Elmer, UK) by the method of Steen and colleagues [15],

using primers 5′-ACCGGGCACCTGTACTCCTCA-3′ and 5′-GCATGAGCTAAGGCACCCAGAC-3′. PCR was performed according to the manufacturer's instructions using Ampliwax® beads to facilitate a hot start and a 100-μl reaction volume with 200 ng genomic DNA, 1xXL reaction buffer, 0.2 mM each dNTP, 0.3 μM each primer, 1.1 mM $Mg(OAc)_2$ and 2 U of the r*Tth*/Vent® DNA polymerase mixture. The XL reaction buffer contains Tricine, K(OAc), glycerol, and DMSO (concentrations not given by the supplier). Cycling conditions were: initial denaturation at 93° C for 1min, 35 cycles at 93° C for 1 min, 65° C for 30 s, 68° C for 5 min, and a final elongation at 72° C for 10 min.

Two cases were positive on both the *CYP2D6*4* and the duplication assays. These were further tested to determine whether the null or the wild type allele was duplicated as described by Sachse *et al.* [10]: a further duplication assay was performed with primers as described by Johansson *et al.* [16], giving a 10kb amplicon in cases positive for a duplication, which was then subjected to a nested PCR followed by digestion with *Hph*I. The primers for the Johansson *et al.* assay were 5′-GCCACCATGGTGTCTTTGCTTTC-3′ and 5′-ACCGGATTCCAGCTGGGAAATG-3′, and we modified the conditions by performing the assay with the Expand Long Template PCR System (Boehringer Mannheim, UK), using a total reaction volume of 25 μl with 2.5 μl of Boehringer buffer 1, 4.5 μl of a 2 mM solution of each dNTP, 0.5 μl of each primer (10 μM solutions), 0.37 μl of the Taq/Pwo enzyme mix, and 200 ng of genomic DNA. Cycling conditions were: initial elongation of 94° C for 2 min; 10 cycles of 93° C for 10 s, 60° C for 30 s, 68° C for 12 min; 20 cycles of 93° C for 10 s, 60° C for 30 s, and 68° C for 12 min with a 15 s increment per cycle; and a terminal elongation step of 68° C for 7 min. The product of this reaction was then diluted 1 : 5 and subjected to nested PCR using primers 5′-TCAACACAGCAGGTTCA-3′ and 5′-CTGTGGT TTCACCCACC-3′. The reaction volume was 65 μl, containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.16 mM each dNTP, 0.1 μM each primer, and 0.2 U AmpliTaq. The cycling conditions were: initial elongation at 94°C for 2 min, 25 cycles of 94°C for 30 s, 58°C for 10 s, 72°C for 1 min, and a final elongation step of 72°C for 7 min. The products were digested with *Hph*I and separated on a 3% agarose gel.

The results were analysed using SPSS for Windows and EpiInfo Version 6 (Centers for Disease Control and Prevention, USA and World Health Organization, Switzerland).

Results

CYP2D6 genotype and deduced phenotype for the 235 subjects refractory to treatment with typical antipsychotics *vs* the 73 responsive to typical antipsychotics are given in Table 1. Of the 235 treatment-refractory subjects, four (1.7%) cases were positive on the duplication assay. However, two cases yielded a *CYP2D6*4/wt* result with the *CYP2D6*4* assay and a positive result with the duplication assay; these cases were found to represent duplications of the *CYP2D6*4* allele. As these cases possessed only one functional copy of *CYP2D6*, they were deduced to be phenotypically equivalent to heterozygous null cases and hence extensive metabolisers, not ultrarapid metabolisers. Therefore only two out of 235 cases (0.9%) were positive for duplication of a wild type allele. In contrast, of the sample of 73 nonrefractory patients, 3 (4.1%) were positive for the duplication assay, of which none was positive for the null alleles tested. The results were therefore in the opposite direction to that predicted by our hypothesis, but they did not reach significance: Fisher's exact test was performed, comparing the presence or absence of UM status in the two clinical groups, which gave a two-tailed *P* value of 0.091. (Chi square was not appropriate here, with the number of cases being less than 5; with Fisher's exact test the one-tailed and two-tailed values were identical, but the value is reported as two-tailed as the results were in the opposite direction to that predicted by the hypothesis).

Table 2 shows the distribution of the alleles with allele frequencies in the two clinical groups, with the results being reported under the assumption that the duplication allele is present in the heterozygous state. If this allele were present in a homozygous state in all cases in which it was found, then the frequencies of the wild type and null alleles in the treatment-refractory group would be unchanged, while that of the duplication allele would be doubled at 0.0085; the frequencies of the wild type, null, and duplication alleles in the nonrefractory group would be 0.71, 0.25, and 0.041, respectively. However, as in the paper by Johansson *et al.* none of their cases with a duplication allele was homozygous for this variant, we have assumed that all of our cases are heterozygous. The frequencies of the *CYP2D6*4*, *CYP2D6*5*, and *CYP2D6*3* alleles, respectively, in the sample of 235 and 73 were: 0.223, 0.024, and 0.018 (in the sample of 235), and 0.219, 0.02, and 0.02 (in the sample of 73). Comparing the presence or absence of the duplication allele in the two groups, Fisher's exact test gave a two-tailed *P* value of 0.10 (Table 2).

Discussion

We did not find an excess of ultrarapid metabolisers in subjects refractory to treatment with typical antipsychotics. On the contrary, only two out of 235 (0.9%) treatment-refractory cases were positive for duplication of a wild-type allele, while three out of 73 (4.1%) nonrefractory

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Table 1 *CYP2D6* genotype and deduced phenotype in subjects refractory to treatment with typical antipsychotics and nonrefractory to treatment with typical antipsychotics; numbers of cases given with percentages in parentheses.

	EM (wt/wt) *or* (wt/mut)	*PM* (mut/mut)	*UM* (*dup of* wt)
Refractory to TAs ($n=235$)	220 (93.6)	13 (5.5)	2 (0.9)*
Non-refractory to TAs ($n=73$)	67 (91.8)	3 (4.1)	3 (4.1)*

TAs, typical antipsychotics, *wt*, wild type, *mut*, nonfunctional mutant or null allele (includes 2 duplications of *mut* in the EM group), dup of *wt*, duplication of wild type allele. *Two-tailed *P*-value for presence or absence of UM status in refractory *vs* nonrefractory group = 0.09 (Fisher's exact test); odds ratio = 0.2, 95% confidence limits 0.02–1.80.

Table 2 Distribution of *CYP2D6* alleles in the treatment-refractory and nonrefractory groups; allele numbers given, with frequencies in parentheses. The *CYP2D6*4 × 2* allele is included in the null alleles.

	Wild type	*Null*	*Duplication of wild type*
Refractory to TAs	343 (0.73)	97 (0.26)	2 (0.0043)
Non-refractory to TAs	103 (0.72)	37 (0.26)	3 (0.021)*

TAs, typical antipsychotics. *Two-tailed *P*-value for presence or absence of duplication allele in refractory *vs* nonrefractory groups = 0.10 (Fisher's exact test); odds ratio = 0.21, 95% confidence limits 0.02–1.88.

cases were genotyped as ultrarapid metabolisers. This gives a trend ($P=0.091$, Fisher's exact test) towards an excess of ultrarapid metabolisers in the nonrefractory group of patients. However, both percentages are within the range for ultrarapid metabolisers in Caucasian populations [2, 3, 6, 10]. Two cases of *CYP2D6*4* duplications were found, which is the second time this has been reported in Caucasians, Sachse and colleagues [10] having provided the first report.

The results demonstrate that ultrarapid hydroxylation by CYP2D6 of typical antipsychotics is not a major cause of failure to respond to treatment with these agents. There are at least five possible explanations for this surprising result. Firstly, we could have failed to find a significant result when there is in fact a significant association of ultrarapid hydroxylation either with treatment-refactory status (the direction of the original hypothesis), or with treatment nonrefractory status (the direction of the trend found). The odds ratio (OR) for UM status, counting the treatment-refractory group as the 'diseased state' and the nonrefractory group as the 'nondiseased state', was 0.2, with exact lower and upper 95% confidence limits of 0.02 and 1.8, respectively (Table 1). This means that ultrarapid metaboliser status is less associated with being treatment-refractory than with being nonrefractory, with the range extending to being more associated with treatment-refractory. Of note the two patient sample groups are unequal in size; if we had had as many in the treatment nonrefractory group and the percentage of ultrarapid metabolisers in this group had remained the same as in our current findings, then we would have found 10 ultrarapid metabolisers in the nonrefractory group, which would have given a chi square of 5.47, a *P* value of 0.019, and an OR of 0.19, with exact lower and upper limits of 0.02 and 0.92, respectively. In this scenario a significant result in the opposite direction to our original hypothesis would have been found.

Secondly, in both of our groups of subjects, the dose of antipsychotic was titrated by the prescribing consultants according to clinical effect. This could obscure any pharmacogenetic effects, i.e. ultrarapid metabolisers could be receiving doses above or at the upper end of the normal prescribed range, and then respond as if they were extensive metabolisers. However, for the nonrefractory group one of the patients with *CYP2D6* duplication was on only 30 mg flupenthixol decanoate 2 weekly (equivalent to 150 mg chlorpromazine daily, i.e. a low dose).

Thirdly, although CYP2D6 is known to contribute to the pharmacokinetics of many typical antipsychotics [1], the specific contribution is different for different antipsychotics, and other cytochromes are involved. CYP2D6 is involved in the first pass metabolism and systemic elimination of perphenazine [17], and the systemic elimination of zuclopenthixol [18]. For these drugs, high CYP2D6 activity would be expected to lead to lower serum levels of the drugs, and hence possible therapeutic resistance. Although inhibition studies demonstrated that CYP2D6 is likely to be involved in the metabolism of chlorpromazine [19, 20], Muralidhan and colleagues [21] showed that CYP2D6 makes a relatively minor contribution to the large interindividual variability seen in plasma chlorpromazine levels.

The systemic elimination of haloperidol has been shown by Llerena and colleagues to be dependent on CYP2D6 activity [22], and although early reports showed that CYP2D6 catalysed the oxidation of reduced haloperidol back to haloperidol [23, 24], other work was not consistent with this [25], and recent reports [26, 27] have demonstrated that CYP3A4 is the primary enzyme involved in this step in the metabolic pathway. The steps

in the metabolism of haloperidol in which CYP2D6 is involved are at present unclear, but, consistent with the results of Llerena and colleagues [22], Nyberg *et al.* showed that a CYP2D6 poor metaboliser had higher concentrations of plasma haloperidol throughout a 4-week treatment period with haloperidol decanoate as compared with 7 CYP2D6 extensive metabolisers [28]. Although none of the subjects in the study of Nyberg *et al.* was an ultrarapid metaboliser, it would be logical to assume that a UM would have low plasma haloperidol levels. Suzuki and colleagues [29] studied the correlation between CYP2D6 genotype and steady-state plasma concentrations (C_{ss}) of haloperidol and reduced haloperidol in a group of 50 Japanese patients with schizophrenia. They found that the mean C_{ss} of haloperidol was significantly higher ($P<0.05$) in the patients with 1 mutant allele compared with those with no mutant alleles, and that the mean C_{ss} of reduced haloperidol was significantly higher ($P<0.05$) in the patients with 1 or 2 mutant alleles compared with those with no mutant alleles. They therefore suggested that the C_{ss} of reduced haloperidol was more dependent upon CYP2D6 activity than the C_{ss} of haloperidol. However, although they did not find a significant difference between the mean C_{ss} of haloperidol in patients with 2 mutant alleles compared to those with no mutant alleles, it is of note that in this study the patients with 2 mutant alleles were either homozygous for the *CYP2D6*10* allele ($n=4$), which is associated with reduced but not absent CYP2D6 activity, or were compound heterozygotes for the *CYP2D6*10* and *CYP2D6*5* alleles ($n=2$). Hence no patient actually had 2 CYP2D6 null alleles. Lane *et al.* [30] examined the relationship between CYP2D6 phenotype (as measured by dextromethorphan/dextrorphan metabolic ratio) and haloperidol disposition in 18 newly hospitalized Chinese patients with schizophrenia. Despite the fact that no PMs were found in this study, significant correlations between the metabolic ratio and plasma haloperidol concentration, reduced haloperidol concentration, and reduced haloperidol/haloperidol ratios were found. In a preliminary report of a larger study, Schmider *et al.* [31] investigated therapeutic drug monitoring data in 178 patients *vs* CYP2D6 genotype and found that PMs had significantly higher reduced haloperidol but not haloperidol concentrations compared with patients with one or no mutant alleles. The suggestion of Suzuki *et al.* [29] that CYP2D6 affects reduced haloeperidol levels at steady-state to a greater extent than haloperidol levels might therefore be correct. However, Suzuki *et al.* also suggested, based on the work of Tyndale and colleagues [24], that CYP2D6 catalyses the oxidation of reduced haloperidol back to haloperidol. As already outlined above, more recent work is not consistent with this [25–27], although the precise step in the metabolism of reduced haloperidol in which CYP2D6 is involved is at present unclear. Young *et al.* [25] showed that reduced haloperidol was the preferred form in the plasma after the administration of a single dose of either haloperidol or reduced haloperidol to healthy volunteers. A negative correlation between clinical response and reduced haloperidol levels or reduced haloperidol/haloperidol ratios has been observed [32]; it is possible that ultrarapid metabolisers of CYP2D6 could have lower reduced haloperidol levels and hence a better clinical response. This would be consistent with the trend that we have found for an excess of UMs in the nonrefractory group. However, Lane *et al.* [30] did not find a correlation between response and reduced haloperidol levels, reduced haloperidol/haloperidol ratios, or haloperidol levels. This is consistent with analyses by other authors [33, 34].

In the case of thioridazine, CYP2D6 catalyses the formation of mesoridazine, a metabolite *with antipsychotic activity* [35], and may be involved in the generation of another active metabolite, sulphoridazine. Extensive metabolisers have been shown to have higher peak levels of mesoridazine and sulphoridazine than poor metabolisers after a single oral dose, with lower levels of thioridazine [35]. The total serum concentrations of substances with antipsychotic activity at steady state will be determined by the relative magnitudes of the equilibrium constants of all the reactions in the metabolic pathway; these equilibrium constants and the relative antipsychotic potencies of the different active metabolites are unknown. It is therefore difficult to predict the effect of ultrarapid metaboliser status on clinical response to thioridazine.

Both of our groups of patients had been treated with various typical antipsychotics; it is therefore possible that we failed to show a correlation in one direction or the other as effects with some antipsychotics *vs* other antipsychotics cancelled each other out. Furthermore, it is possible that some patients in the treatment-refractory group were treated with agents whose levels are not significantly affected by CYP2D6 genotype (such as chlorpromazine). However, we would emphasize that a significant result would be unlikely to be obscured by either of the above possibilities as the numbers of individuals with duplications is *very low in both* the treatment-refractory and nonrefractory groups. It is also of note that the subject on zuclopenthixol who is an ultrarapid metaboliser is clinically stable on a low dose.

Fourthly, several of the above studies (especially those on normal volunteers) were single-dose pharmacokinetic analyses; single dose effects may differ markedly from those seen at steady state in a situation of pharmacological adaptation [36]. However, the work of Nyberg *et al.* [28], Suzuki *et al.* [29], Lane *et al.* [30], and Schmider *et al.* [31] was conducted on patients at steady-state. Furthermore, Jerling *et al.* [37] conducted a study on

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patients during continuous treatment and CYP2D6 genotype was shown to predict significantly the oral clearance of perphenazine and zuclopenthixol (patients with 2 CYP2D6 null alleles having a significantly lower clearance than those with one or no mutant alleles).

Finally, other factors may contribute towards nonresponse to medication, including noncompliance, pharmacodynamic factors, other biological factors, and psychosocial factors. Non-compliance occurs in up to 50% of patients on neuroleptics [38]. Pharmacodynamic factors have been implicated in the clinical response to clozapine, an atypical antipsychotic [39]; it may be that other pharmacodynamic factors (e.g. D_2-receptor variants) are involved in the response to typical antipsychotics. Lieberman and colleagues [40] and Van Os *et al.* [41] have reviewed predictors of outcome in psychotic illness and concluded that factors such as longer duration of untreated illness, and structural brain abnormality on CT or MRI predict unfavourable outcome, while living in a low 'expressed emotion' environment is one of the predictors of a favourable outcome.

We have not found an association between ultrarapid metaboliser status and being treatment-refractory to typical antipsychotics. On the contrary, we found a trend towards an association between ultrarapid metaboliser status and being nonrefractory to typical antipsychotics, which could have reached significance had our nonrefractory group been equal in size to our refractory group.

Nonetheless, we have not excluded the possibility that ultrarapid metaboliser status could lead to failure to respond to a standard dose of some typical antipsychotics. But as only 0.5% to 7% of Caucasians are ultrarapid metabolisers, unless ultrarapid metaboliser status were associated with the psychotic illnesses for which the antipsychotics were prescribed (in this study, mainly schizophrenia), then as treatment resistance occurs in up to 30% of cases of schizophrenia, this factor would be unlikely to account for the majority of cases of treatment resistance. In summary, our data are not consistent with ultrarapid metaboliser status being a major cause of failure to respond to typical antipsychotics, and are consistent with ultrarapid metaboliser status being weakly associated with response to typical antipsychotics, especially in the case of haloperidol.

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Identification of novel polymorphisms in the 5′ flanking region of *CYP1A2*, characterization of interethnic variability, and investigation of their functional significance

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CYP1A2 activity has been demonstrated to be bimodally or trimodally distributed in several populations, consistent with a codominant or recessive functional genetic polymorphism. However, studies aimed at identifying polymorphisms in *CYP1A2* have not yet adequately accounted for this distribution pattern. To search for functional polymorphisms, we performed genome-walking, polymerase chain reaction (PCR) sequencing, and cloning, and identified three novel polymorphisms in the 5′ flanking region of *CYP1A2*: a $T_{-3591}G$ substitution, a $G_{-3595}T$ substitution, and a T_{-3605} insertion. The frequency of the $T_{-3591}G$ substitution was determined by a PCR-restriction fragment length polymorphism assay, and found to be significantly higher ($P < 0.0001$) in Taiwanese (allele frequency 0.128, $n = 125$) compared to Caucasians (0.017, $n = 87$) or African Americans (0.024, $n = 104$). The functional consequence of the $T_{-3591}G$ and the $G_{-3595}T$ substitutions was determined by site-directed mutagenesis followed by transient transfection experiments. The $T_{-3591}G$ mutation was shown to be nonfunctional, while although the $G_{-3595}T$ mutation appeared to result in an increase in promoter activity, this was only to a small degree and therefore unlikely to be important *in vivo*. In addition, we report 532 bases of 5′ flanking sequence further upstream than that reported to date, and four sequence discrepancies compared to the original published sequence ($G_{-3649}C$, ΔT_{-3650}, ΔA_{-4072}, and C_{-4093} ins). Pharmacogenetics 10:695–704 © 2000 Lippincott Williams & Wilkins

Keywords: cytochrome P450, CYP1A2, promoter, pharmacogenetics, genotype.

Introduction

The cytochrome P450 enzyme CYP1A2 plays a major role in the metabolism of many commonly used drugs, including chlorpromazine, trifluoperazine, clozapine, olanzapine, tricyclic antidepressants, zopiclone, tacrine, paracetamol, xanthines including caffeine, and lignocaine (Imaoka *et al.*, 1990; Aitchison *et al.*, 2000). It is also involved in the metabolism of the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which produces a parkinsonian syndrome in man (Coleman *et al.*, 1996), and in the activation of arylamines and heterocyclic amines implicated in the genesis of colon and bladder cancer (McManus *et al.*, 1990; Boobis *et al.*, 1994; Eaton *et al.*, 1995; Hammons *et al.*, 1997).

There is wide interindividual variation in CYP1A2 activity (Kalow & Tang, 1991a) which, in most studies, has been demonstrated to be trimodally or bimodally distributed (Butler *et al.*, 1992; Lang *et al.*, 1994; Nakajima *et al.*, 1994; Schrenk *et al.*, 1998;

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Throughout this paper, nucleotides are numbered with the start of translation as +1 (position 2804 in GenBank accesssion number M31664) in accordance with the international nomenclature for human CYP1A2 alleles, available at www.imm.ki.se/CYPalleles.

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Ou-Yang *et al.*, 2000). Butler *et al.* (1992) studied individuals from Arkansas, Italy, and China, and found that CYP1A2 activity was trimodally distributed, with the range of percentages in the three populations being: 12–13% slow, 51–67% intermediate, and 20–37% rapid metabolizers. Nakajima *et al.* (1994) studied the pattern of CYP1A2 activity in eight pedigrees, the results of which were consistent with genetic polymorphism at a single gene locus, with autosomal codominant transmission. At least some of the interindividual variability in CYP1A2 activity is, however, explicable by environmental factors. The enzyme is inducible by various dietary substances, drugs, and toxins, and including cruciferous vegetables, heterocyclic amines, polycyclic aromatic hydrocarbons (e.g. 3-methylcholanthrene), and heterocyclic aromatic hydrocarbons (including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, or TCDD), caffeine, cigarettes, paracetamol, omeprazole, and carbamazepine (Rost *et al.*, 1994; Parker *et al.*, 1998; Aitchison *et al.*, 2000). CYP1A2 may be inhibited by lutein (found in leafy green vegetables, Le Marchand *et al.*, 1997), grapefruit juice (Fuhr *et al.*, 1993), oestrogens and pregnancy (Knutti *et al.*, 1981; Rietveld *et al.*, 1984; Abernethy & Todd, 1985; Vistisen *et al.*, 1992; Le Marchand *et al.*, 1997), quinolone antibiotics (Fuhr *et al.*, 1992), fluvoxamine (Brøsen *et al.*, 1993), and, in smokers, heavy ethanol consumption (Rizzo *et al.*, 1997). Nonetheless, in a study of CYP1A2 activity in 786 Caucasians, Tantcheva-Poór *et al.* (1999) found that 63% of the overall variation remained unaccounted for after analysis for the major covariates (e.g. coffee consumption). This finding points to the existence of other factors, such as genetic polymorphisms, as contributors to the variation in CYP1A2 activity.

There is evidence for interethnic variation in CYP1A2 activity. Butler *et al.* (1992) found that the frequency of rapid metabolizers in nonsmokers was 20% in 50 Italians and 37% in 77 individuals from Arkansas. Le Marchand *et al.* (1997) found a significantly higher mean CYP1A2 activity in 15 Caucasians compared to 45 Japanese. Relling *et al.* (1992) showed that CYP1A2 activity was significantly lower in a group of 63 black subjects in comparison to a group of 246 white subjects. Lang *et al.* (1994) found African American smokers had CYP1A2 activity approximating that of African American or Caucasian non-smokers. Furthermore, a study of clozapine levels in 162 Taiwanese found mean plasma concentrations 30–50% higher than those reported in Caucasians for equivalent doses (Chang *et al.*, 1997).

Nakajima *et al.* (1994), using polymerase chain reaction (PCR) followed by direct sequencing, screened the exons, exon–intron junctions, and the 5′ flanking region to −3470 bp in two non-smoking poor metabolizers, 1 non-smoking extensive metabolizer, 1 smoking poor metabolizer, and 1 smoking extensive metabolizer and found no differences in the nucleotide sequence between each phenotype. Given the indication that factors including genetic polymorphism in *CYP1A2* could contribute to CYP1A2 variability and this negative finding, we decided to screen the 5′ flanking region of *CYP1A2* further upstream than −3470 bp. TCDD induces the expression of *CYP1A1* as well as that of *CYP1A2*, and in this case, has been shown to exert this effect via its interaction with xenobiotic responsive elements (XREs) in the promoter sequence. Two XRE-like sequences have been identified in the human *CYP1A2* promoter, at positions −3382 to −3400, and −3037 to −3055 (Quattrochi *et al.*, 1994). These sequences were shown to lie within regions that contribute to the induction of *CYP1A2* by 3-methylcholanthrene (Quattrochi *et al.*, 1994), and in transient transfections, TCDD was found to induce the *CYP1A2* promoter (Postlind *et al.*, 1993). However, the fold induction is much higher for *CYP1A1* than for *CYP1A2* (using a *Kpn*I fragment of *CYP1A2*, −4096/−842), and induction of *CYP1A2* was not seen in stable transfectants (Postlind *et al.*, 1993). We therefore decided to determine the sequence of the *CYP1A2* 5′ flanking region that was further upstream than −4096, to see whether or not another XRE could be found.

Materials and methods

Study design

Three sample groups were used for this study. The first was 87 Caucasians from the UK. The second was 125 Taiwanese, the third was 104 African American subjects; the DNA from both of these latter groups has been described previously (Wei *et al.*, 1998). Approval for the study was obtained from the Maudsley and Bethlem Royal Hospitals/Institute of Psychiatry and King's College Hospital, London (UK) Ethical Committees.

Genome walking

As the original aim of the study was to search for functional mutations in the 5′ flanking region of *CYP1A2* beyond −2.6 kb, primers were designed for use with the Genome Walker Kit (Clontech Laboratories Inc., CA, USA). Gene-specific primers cyp1abr1 and cyp1abr2 (Table 1) were used together with the supplied adaptor primers, according to the manufacturer's instructions in the primary and secondary

Table 1. Primers and annealing conditions used for genome-walking from, polymerase chain reaction amplification of, and site-directed mutagenesis of the *CYP1A2* 5′ flanking region. Underlined nucleotides are the mutant sites in the primers used for site-directed mutagenesis

Location of primers		*Sequence*	*Annealing conditions*
Cyp1abr2	−3387 → −3411	5′-GTGCGTGTCAGGTCTCTTCACTGTA-3′	72 °C, 3 min and 67 °C, 3 min (see Genome Walker kit manual)
Cyp1abr1	−3292 → −3316	5′-GGTTAGGTGCCATTCTCGTCACATC-3′	
M2	−3798 → −3820	5′-GCTGGAATTACAGGTGTGCACCA-3′	55 °C, 1 min
M1	−3545 → −3562	5′-CTCCTGTATGACAGACTA-3′	
Cyp1abf	−3880 → −3899	5′-GTGCAGTGGTGCGATCTTGG-3′	54 °C, 30 s (with cyp1abr2)
M3	−3454 → −3472	5′-GGAAGTCACTATGCACAGC-3′	55 °C, 1 min (with M2)
M4	−3706 → −3726	5′-GAACTCCTGGCCTCACTCAAG-3′	53 °C, 30 s (with M3)
M5	−3633 → −3651	5′-GTCCCAGCTGACATATGCA-3′	53 °C, 30 s (with cyp1abr2)
SDM1F	−3568 → −3616	5′-CCTGTAATTTAATTTTTTTAAGTTTGAAGAAAACATTAAAAATAAAAAG-3′	55 °C, 1 min
SDM1R	−3568 → −3616	5′-CTTTTTATTTTTAATGTTTTCTTCAAACTTAAAAAAATTAAATTACAGG-3′	
SDM2F	−3583 → −3627	5′-CTATATTGTATCCTGTAATTTAATTTTTTTAATTTTTAAGAAAAC-3′	55 °C, 1 min
SDM2R	−3583 → −3627	5′-GTTTTCTTAAAAATTAAAAAAAATTAAATTACAGGATACAATATAG-3′	
SDM3F	−3583 → −3627	5′-CTATATTGTATCCTGTAATTTAATTTTTTTAATTTTGAAGAAAAC-3′	55 °C, 1 min
SDM3R	−3583 → −3627	5′-GTTTTCTTCAAAATTAAAAAAAATTAAATTACAGGATACAATATAG-3′	

PCR reactions on the five 'libraries' of uncloned, adaptor-ligated genomic DNA fragments. (These five 'libraries' are produced by digestion of genomic DNA with five different enzymes: *Eco*RV, *Sca*I, *Dra*I, *Pvu*II, and *Ssp*I.) The secondary PCR products were purified using QIAquick PCR purification (Qiagen Ltd, Crawley, West Sussex, UK), and sequenced directly using 60–90 ng of purified PCR product, standard cycle sequencing with primer cyp1abr2, ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosytems, UK), and an ABI 377 DNA sequencer.

PCR sequencing

DNA was extracted from whole blood samples using standard procedures. Primers M1 and M2 (Table 1) were used in a final volume of 100 µl with 10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.001% (w/v) gelatin, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.5 µM each primer, and 2.5U AmpliTaq (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). PCR products were purified using a Wizard PCR purification kit (Promega Corporation, Madison, WI, USA), and subjected to cycle sequencing using primer M1. Sequence comparison was undertaken with the program MacVector (Oxford Molecular Limited, Oxford, UK).

Further mutation identification and cloning

The PCR product of reaction cyp1abr2-M5 of African American sample number 9 was cloned using the TA Cloning Kit (Invitrogen Corporation, Carlsbad, CA, USA) with the vector pCR2.1. The protocol for colony lysis and PCR was then followed from the PCR-Trap manual (GenHunter Corporation, Nashville, TN, USA), using primers M13R (5′-CAGGAAACAGCTATGAC-3′) and T7 (5′-TAATACGACTCACTATAGGG-3′) with annealing at 50 °C for 30 s. Clones positive for the insert were subjected to sequencing using T7.

The PCR product of reaction M3-M4 of African American sample number 43 was also cloned using the TA cloning kit as above. Because sample 43 had already been shown to be heterozygous for the $T_{-3591}G$ substitution, we screened PCR products from the clones using *Mbo*II digestion, and sequenced products that did and did not digest using T7.

PCR-restriction fragment length polymorphism (RFLP) test

A PCR-RFLP test for the $T_{-3591}G$ substitution was designed. This involved a nested PCR, the first PCR reaction using primers cyp1abr2 and cyp1abf, the second PCR reaction using primers M2 and M3 (Table 1). Products were digested with *Mbo*II (New England Biolabs, Beverly, MA, USA) and separated on a 3% agarose gel.

Site-directed mutagenesis

To investigate the functional significance of the $T_{-3591}G$ and $G_{-3595}T$ substitutions, site-directed mutagenesis was performed using the QuikChange Site-directed mutagenesis kit (Stratagene Inc., La Jolla, CA, USA) on the pL1A2N plasmid that had already been constructed by LQ. This plasmid is approximately 10 kb in length, and contains a *Kpn*I fragment of *CYP1A2* (-4096/-842) proximal to a luciferase reporter gene (Postlind *et al.*, 1993).

Site-directed mutagenesis to create the $T_{-3591}G$ mutation was performed with primers SDM1F and SDM1R (Table 1). Products were digested with *Dpn*I, and Epicurian Blue cells transformed. Cells were plated on to CircleGrow (BIO 101 Inc., Vista, CA, USA) agar plates with ampicillin at 50 ng/ml, the pUC18 control reaction being plated in NZY+ broth. Plasmid was harvested from cultures grown from single colonies using QIAGEN-tips 500 (QIAGEN Inc., Santa Clarita, CA, USA). DNA was quantified using a spectrophotometer. The mutant plasmid was subjected to sequencing using primer M3, which confirmed correct introduction of the mutation, and absence of any other differences in DNA sequence from the parent plasmid (data not shown). Sequence comparison was undertaken with the programme Hitachi MacDNASIS Pro version 3.4 (Novex, San Diego, CA, USA). This plasmid was then termed SDM1.

Site-directed mutagenesis to create the $G_{-3595}T$ mutation was performed in a similar manner to the above, using primers SDM2F and SDM2R, and the resultant plasmid was designated SDM2. A third site-directed mutagenesis reaction was performed in order to create a double mutant, with both the $T_{-3591}G$ and the $G_{-3595}T$ substitutions, using primers SDM3F and SDM3R, the resultant mutated plasmid being designated SDM3.

Transient transfections

HepG2 cells were seeded at 1.25×10^5/ml in 6-well plates in complete growth medium with 10% serum. The complete growth medium consisted of Dulbecco's Modified Eagle Medium and F-12 Nutrient Mix in a 1:1 ratio (DMEM/F12, 1:1, GIBCO BRL, Life Technologies Inc., Gaithersburg, MD, USA), with 10 mM Hepes, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were grown up for 24 h at 37 °C under 5% CO_2, and transfected the next day with 2 μg plasmid and 0.5 μg CMVβ (ClonTech Laboratories Inc., Palo Alto, CA, USA), using lipofectamine reagent (Life Technologies), with a 1:8 ratio (mass:-mass) of DNA:lipofectamine and OPTI-MEM I (Life Technologies) as the serum-free medium. The CMVβ plasmid expresses β-galactosidase from the human cytomegalovirus immediate early gene promoter. At 40 h post transfection, one 6-well plate for each plasmid was treated with 10 nM TCDD. Sixty hours post transfection, the cells were harvested for luciferase activity, using a Luciferase Assay System with Reporter Lysis Buffer (Promega Corporation, Madison, WI, USA). The lysates were cleared by centrifugation for 2 min at 4 °C, and 20 μl of cleared lysate was then assayed for luciferase activity using a Lumat LB9501 luminometer (Berthoid, Sci West, Arvada, CO, USA). Lysates (50 μl) were assayed in duplicate for β-galactosidase activity using the β-galactosidase Enzyme Assay System with Reporter Lysis Buffer (Promega Corporation, Madison, WI, USA).

BAC clone generation and sequencing

A human bacterial artificial chromosome (BAC) library (Genome Systems Inc., St Louis, MO, USA) was screened with a *Bam*HI/*Pst*I fragment (–808/–10, in intron 1). The probe was labelled by random priming with DNA polymerase I, large (Klenow) fragment using ^{32}P-dCTP (Ready To Go Kit, Pharmacia Biotech Inc., Piscataway, NJ, USA). Labelled probe was added to membranes at 2.0×10^6 c.p.m./ml in standard hybridization buffer, and hybridized at 42 °C overnight. Membranes were washed in 0.1 × SSC, 0.5% sodium dodecyl sulphate, and exposed to autoradiographic film overnight at −70 °C. This procedure generated three positive BAC clones. These clones were then further probed with the following oligonucleotides: 5′-TCTGCCATCTTCTG CCTGGTATTCTG-3′ (exon 2, positions 52–77), 5′-TCTTCCTCTTCCTGGCCATCCTGCTAC-3′ (exon 7, positions 5238–5264), and 5′-TGGCAGAGCTCTTCCT CATGTGTGCAG-3′ (5′ flanking region, positions −1217 to −1245). Two out of the three clones were positive on all three hybridizations and were used in further studies (BAC7 and BAC8). The presence of *CYP1A2* in these clones was further confirmed by both PCR using primer pair cyp1abr2-cyp1abf and sequencing with primer M1. Clones BAC7 and BAC8 were then subject to direct sequencing using primer CYP1A2.5R (5′-AGCTCGATCATGTGTAGCTTG-3′) and BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Applied Biosystems). A further primer, CYP1A2.5R2 (5′-CATCTTG CAGTGGTGTAAT-3′), was designed from the resultant sequence, and the clones were subject to direct sequencing with this primer.

Statistical analysis

The results for the $T_{-3591}G$ polymorphism in the three ethnic groups were analysed by EpiInfo Version 6 (Centers for Disease Control and Prevention, USA,

and World Health Organization, Switzerland). The transfection data were analysed using SigmaStat 2.0 (SPSS Inc., Chicago, IL, USA).

Results

Genome-walking, PCR sequencing, and cloning

Sequence from the secondary PCR product of the *Sca*I 'library' of the Genome Walker kit produced sequence which aligned with the 5′ flanking sequence of *CYP1A2*, and revealed the following sequence discrepancies compared with the published sequence: a $G_{-3649}C$ substitution and a T_{-3650} deletion. We investigated these potential polymorphisms by PCR sequencing using PCR reactions M2–M3 with sequencing primers M1 and M2, and found the same sequence discrepancies in 11 Caucasians and eight Taiwanese samples. Furthermore, these discrepancies were found in plasmid pL1A2N supplied by LQ, which is a derivative of plasmid pH4CAT1, the latter being the plasmid that was sequenced by Quattrochi and Tukey (1989), and hence these sequence discrepancies are likely to represent publication errors.

However, the above PCR sequencing also revealed a $T_{-3591}G$ substitution in one of the Caucasians and one of the Taiwanese. Further PCR sequencing using PCR reactions M3–M4 or cyp1abr2-M5 with sequencing primers M1, M3, or M5 also revealed a second polymorphic site in the 5′ flanking region of *CYP1A2*, a $G_{-3595}T$ substitution, in one out of a further 20 Caucasians subjects, three out of a further 31 Taiwanese subjects, and three out of 10 African American subjects sequenced. The Caucasian and the three Taiwanese subjects were heterozygous for both of the point mutations (giving an allele frequency of 0.025 in the Caucasians, and 0.048 in the Taiwanese), while two of the African American subjects were homozygous for the $G_{-3595}T$ substitution (and wild-type for the T_{-3591}), and one was heterozygous for the $G_{-3595}T$ substitution (and wild type T_{-3591}), which gives an allele frequency of 0.25 in this subgroup. The numbers of samples in the three populations for which we have data on both the $T_{-3591}G$ and $G_{-3595}T$ polymorphisms are too small to enable calculation of whether or not the two polymorphisms are in linkage disequilibrium.

For two of the African American subjects, sequence in the reverse direction was unambiguous at the 3′ end but from point −3605 had the appearance of two sequences superimposed upon each other, indicating heterozygosity for a single base insertion or deletion at that point. This was resolved by cloning and sequencing the PCR product, which revealed a T insertion at −3605 in some clones from African American samples numbers 9 and 43. For sample number 43, *Mbo*II digestion showed that the T_{-3605} insertion and the $T_{-3591}G$ substitution occurred on different alleles. The T_{-3605} insertion was therefore found in heterozygous state in two out of 10 African Americans sequenced (giving an allele frequency of 0.10), in 0 out of 31 Taiwanese sequenced, and in 0 out of 20 Caucasians sequenced.

PCR-RFLP testing

We used a PCR-RFLP test to determine the frequency of the $T_{-3591}G$ substitution in 87 Caucasians, 125 Taiwanese and 104 African Americans (Fig. 1). (These included 12 of the Caucasians sequenced as above, and all of the sequenced Taiwanese and African Americans.) DNA from all subjects that appeared to be heterozygous for the $T_{-3591}G$ substitution on PCR-RFLP analysis was sequenced using PCR reactions M3–M4 or cyp1abr2-M5 and primers M1, M3, or M5, in order to confirm genotype. This revealed interethnic variability in the frequency of the polymorphism (Table 2), the frequency of the G_{-3591} allele being much higher (0.128) in the Taiwanese as compared to that in the Caucasians (0.017) or the African Americans (0.024). This difference is highly significant ($P < 0.0001$). The genotype frequencies in each population were in Hardy–Weinberg equilibrium. Screening for the remaining two polymorphisms was felt to be inappropriate before their functional significance was known.

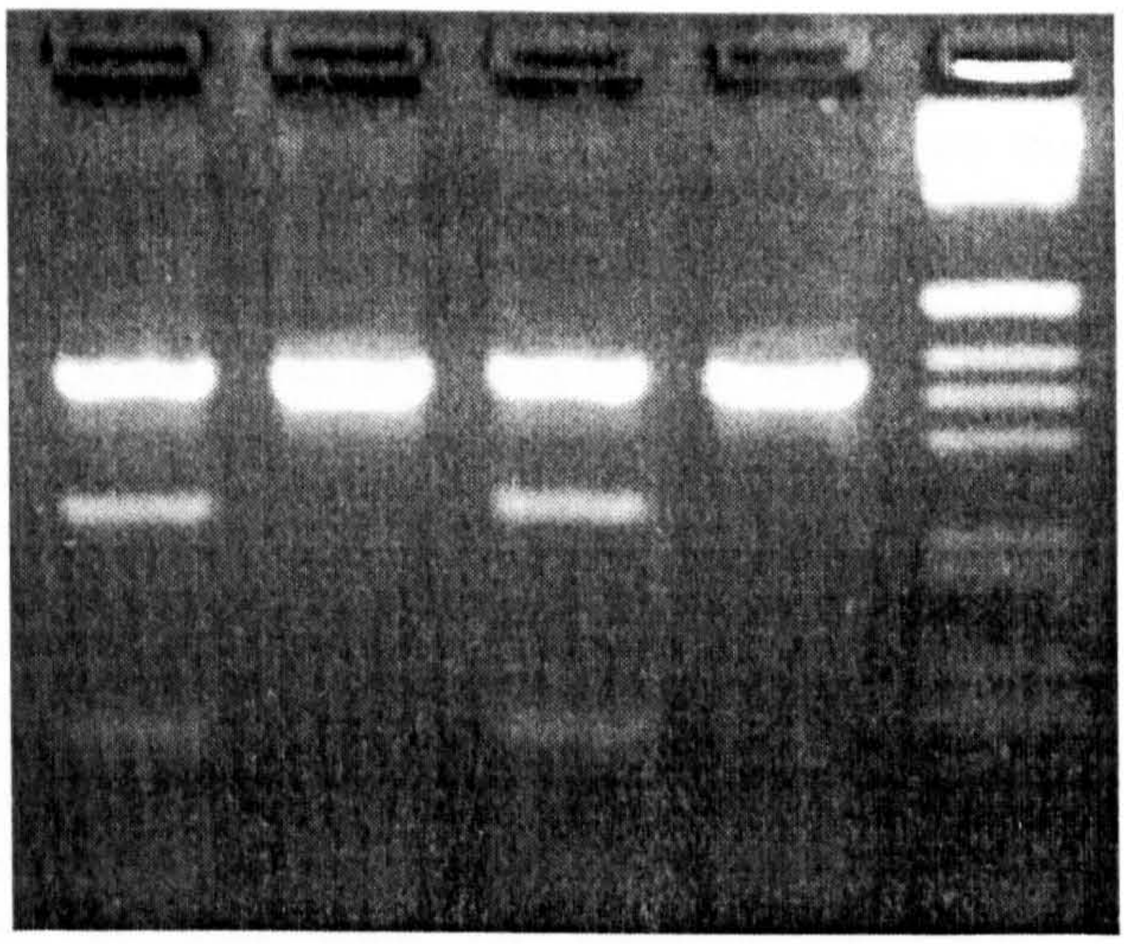

Fig. 1. Agarose gel electrophoresis of *Mbo*II digestion of M2–M3 PCR products. Subjects in lanes 1 and 3 are heterozygous for the $T_{-3591}G$ substitution; subjects in lanes 2 and 4 are homozygous wild-type; in lane 5 is a 1 kb ladder.

Table 2. *CYP1A2* $T_{-3591}G$ genotypes in 87 Caucasians, 104 African Americans, and 125 Taiwanese

	Homo mut	*Het mut*	*Homo wt*
Caucasian	0	3	84
African American	0	5	99
Taiwanese	4	24	97

Mut, mutant, G_{-3591} allele; *wt*, wild-type, T_{-3591} allele. Analysis by genotype, comparing homozygous mutant or heterozygous mutant versus wild-type, Caucasians versus Taiwanese: $\chi^2 = 14.8$, $P = 0.0001$. By allele, comparing Caucasians versus Taiwanese: $\chi^2 = 16.6$, $P < 0.0001$. For African Americans versus Taiwanese by genotype, $\chi^2 = 14.2$, $P = 0.0001$ and by allele $\chi^2 = 16.5$, $P < 0.0001$. For Caucasians versus African Americans, by genotype and by allele, Fisher's exact test gives a one-tailed *P*-value of 0.46.

Transient transfections

We chose to investigate the functional significance of the $T_{-3591}G$ and $G_{-3595}T$ mutations as these were the more frequent of the mutations identified. The results of the transient transfection experiments using HepG2 cells showing the constitutive (non-induced) levels of luciferase (reporter product) activity and induced (TCDD-treated) levels are shown in Fig. 2.

Comparing the constitutive levels of luciferase activity, Bartlett's test for homogeneity of variances gave a Bartlett statistic of 23.26, and $P < 0.0001$. A non-parametric (Kruskal–Wallis) test was therefore used, which gave a Kruskal–Wallis statistic of 30.52, with three degrees of freedom, and $P < 0.001$, i.e. showing a significant difference between the median values of the four groups. Dunn's Multiple Comparisons Test, comparing the results of each of the other groups of transfections versus the wild-type (pL1A2N) gave a significant difference between SDM2 ($G_{-3595}T$) and wild-type ($P < 0.05$). On visual inspection of the data (Fig. 2), it can be seen that the SDM2 group appears to have a higher mean luciferase activity than the pL1A2N group. However, the magnitude of this difference is small (0.72 relative light units per milliunit of β-galactosidase $\times 10^{-3}$), giving a 1.2-fold increase in the mean value for the SDM2 group compared to the pL1A2N group. It is possible that this translates into a significant functional difference *in vivo*, but unlikely.

Comparing the mean fold induction for each transfection experiment performed for the four plasmids using the Kruskal–Wallis test, gave a Kruskal–Wallis statistic of 4.99, and a *P*-value of 0.17. This means that there is not a significant difference between the fold induction in the four groups (mean induction 2.6-fold).

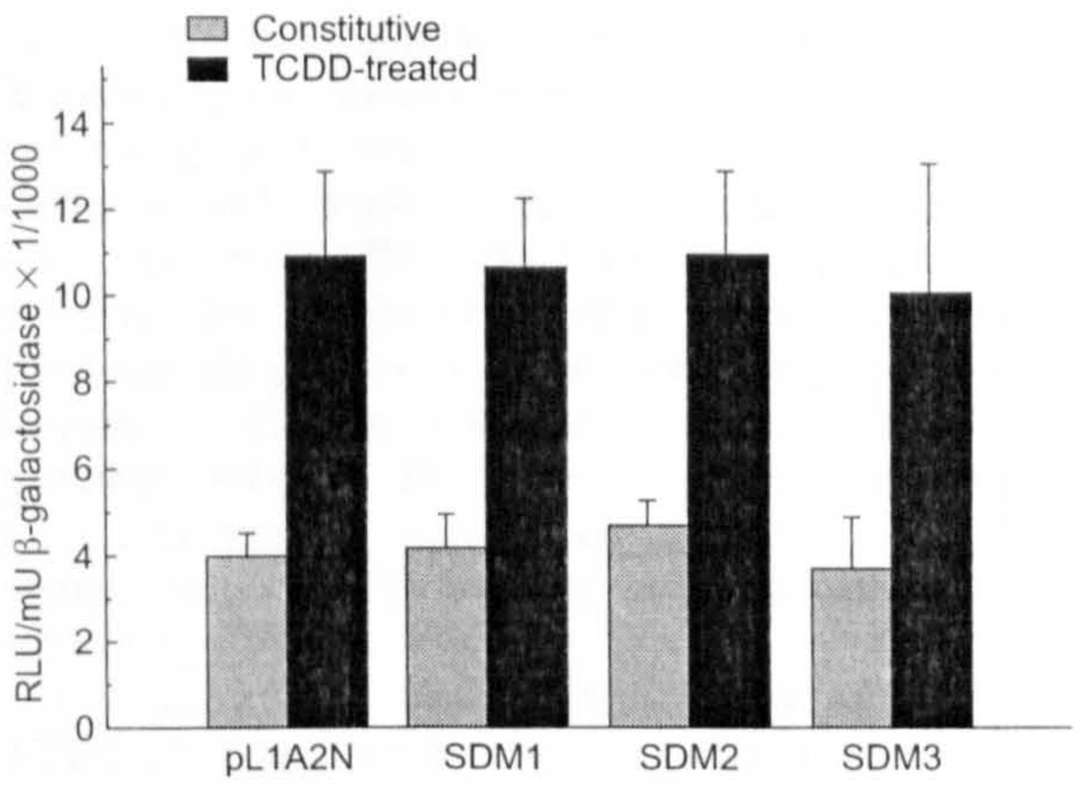

Fig. 2. Results of transient transfections of HepG2 cells with luciferase reporter vectors containing the *CYP1A2* promoter; pL1A2N is the wild-type promoter; SDM1, SDM2, and SDM3 were created by site-directed mutagenesis of pL1A2N. SDM1 contains the $T_{-3591}G$ substitution, SDM2 the $G_{-3595}T$ substitution, and SDM3 both the $T_{-3591}G$ and the $G_{-3595}T$ substitutions. Cells were cotransfected with a CMVβ plasmid (expressing β-galactosidase); results are given as relative light units (RLU) per milliunit (mU) of β-galactosidase $\times 10^{-3}$. The TCDD-treated groups were treated with 10 nM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin at 40 h post transfection for 20 h. Mean values with standard deviations of 3–4 transfections per group, each with 6–12 replicate wells, are shown.

Direct sequencing of BAC clones

Sequencing of *CYP1A2* positive BAC clones was undertaken in order to determine the sequence of the *CYP1A2* 5′ flanking region further upstream than that previously published, in order to search for another XRE in this sequence. Sequencing of the cyp1abr2–cyp1abf PCR product from clones BAC7 and BAC8 with primer M1 was identical to the expected published sequence except for the $G_{-3649}C$ substitution and the T_{-3650} deletions already identified as above, thus confirming the presence of *CYP1A2* in these clones. Direct sequencing of BAC7 and BAC8 with CYP1A2.5R and CYP1A2.5R2 produced the sequence shown in Fig. 3. Both sequences differed from that published by Quattrochi and Tukey (1989) in having an A_{-4072} deletion, and a C_{-4093} insertion (the latter shifting the *Kpn*I site further 5′ by one nucleotide). We checked this region of pL1A2N (sequenced using primer M3), and found that this plasmid also had these sequence discrepancies compared with the original published sequence. The discrepancies therefore represent publication errors. We searched the 532 bp of novel sequence for

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−4626      TGGGGTTCAAAGGATTCTCTTGCATCAGCCTCCTGAGTAGCTGGGACTACAGGCAT
−4570  GCGCCACCATGGTCGGCTAATTTTTTTGTATTTTTAGTAGAGATGGGGTTTTATCATGTT
−4510  GGCCAGGCTGGTCCAGAACTCCTGACCTCAAGTGATCCGCCCACATCAGCCTCCCAAAGT
−4450  GCTGAGATTACAGATGTGAGCCTCCACACCAGGCCTGAGACTCTGTCTCAAAACAACAAC
−4390  AACAACAACCAGAATGTATCAATATTCATCCACGAATTGTAACAAATATATTACACCACT
−4330  GCAAGATGTTAATAATAGGGGAAACTGCAGAGTGGGGGTGGTAAATGGCCACTTTTACCT
−4270  CCCTCATCATACTTTCCACTCAATTTTTCTGTGAACCAAAGACTGCTCTAAAAAAATCTA
−4210  TTAGCTTTTTAAAATTCCTTGGCTCCCCTCCAAAAAGTGTACATATGACATGATCTCATT
−4150  TATGTAAAATACAACAAGCAAAACAAATCCATGCAATAGATGTTGGGGTCATGGGTACCC
−4090  TTGAGAAAGGAACACAAC-GGGACTTCTTGGATG
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Fig. 3. Nucleotide sequence of the *CYP1A2* 5′ flanking region further 5′ than that yet reported. Sequence from −4626 to −4098 represents novel sequence. Discrepancies as compared with the sequence reported by Quattrochi & Tukey (1989) are shown: the C_{-4093} insertion is underlined, and the A_{-4072} deletion is illustrated.

XRE sites using MatInspector Version 2.2 (Quandt *et al.*, 1995), and found no XRE motif.

Discussion

We have found three novel polymorphisms in the *CYP1A2* 5′ flanking region: a $T_{-3591}G$ substitution, a $G_{-3595}T$ substitution, and a T_{-3605} insertion. A PCR-RFLP test for the $T_{-3591}G$ polymorphism was used to genotype 87 Caucasians, 104 African Americans, and 125 Taiwanese (Table 2); this revealed a significantly higher frequency of the G_{-3591} allele in Taiwanese compared to Caucasians and African Americans.

The results of transfection experiments (Fig. 2) showed that the SDM2 ($G_{-3595}T$) group had significantly higher constitutive activity than wild-type, but only by a factor of 1.2. This small increase in promoter activity is unlikely to be of functional significance *in vivo*. Of note, if the $G_{-3595}T$ substitution were associated with increased promoter activity, the direction of effect would be in the opposite direction to that predicted by the work of Chang *et al.* (1997), whose data are consistent with the existence of a polymorphism causing reduced CYP1A2 activity present at a higher frequency in Taiwanese compared to Caucasians. We performed a search for potential transcription factor binding sites in the region of interest using MatInspector Version 2.2 (Quandt *et al.*, 1995), which showed that both the $T_{-3591}G$ and $G_{-3595}T$ polymorphisms lie within the putative binding site for CCAAT/enhancer binding protein β (C/EBPβ, −3582 to −3595 on forward strand). C/EBPβ is part of a family of transcription factors, that are widely expressed (Lekstrom-Himes & Xanthopoulos, 1998), may bind to a variety of sites including activating protein-1 sites, may heterodimerise with other transcription factors including c-*fos*, c-*jun*, and ATF-2, and has been shown to play a crucial role in the regulation of the expression of liver-specific genes (e.g. phosphoenolpyruvate carboxykinase or PEPCK; Croniger *et al.*, 1998). It is of possible interest that two of the polymorphisms ($T_{-3591}G$ and $G_{-3595}T$) that we have identified lie within the C/EBPβ binding site, and that the third one (T_{-3605}ins) is close.

Comparison of the TCDD-induced activity of the *CYP1A2* promoter in the four plasmids revealed no significant difference (mean induction 2.6-fold for the whole group). In the case of PEPCK regulation, C/EBP binding to a specific domain, P3(I), is required for the liver-specific (i.e. constitutive) expression of PEPCK, whereas its binding to two other domains, the cAMP regulatory element (CRE) and the glucocorticoid response unit (GRU) is involved in the induced expression. By analogy, it is therefore possible that this upstream C/EBPβ binding site is involved in the regulation of constitutive but not inducible *CYP1A2* expression, but, again, we feel that a rigorous interpretation of our data does not support this.

The functional significance of the T_{-3605} insertion was not tested. However, although this lies within the putative binding sequence of a human transcription factor (octamer factor 6, −3601 to −3614 on the forward strand or −3597 to −3613 on reverse strand), the T_{-3605} insertion does not alter its core recognition sequence. Taken together with the fact that the other two nearby polymorphisms do not appear to cause a significant functional alteration in *CYP1A2* promoter activity, we feel that it is also unlikely that this polymorphism would lead to a change in *CYP1A2* promoter activity. Nonetheless, even though our polymorphisms appear to be non-functional, they may be useful in disease association studies, provided that the interethnic variation in frequency is taken into account.

Genome walking and sequencing also revealed two other sequence discrepancies compared with the sequence published by Quattrochi and Tukey (1989): a $G_{-3649}C$ substitution and a T_{-3650} deletion. These sequence changes have since been reported by Nakajima *et al.* (1999). [In Figure 1 of Nakajima *et al.* (1999), they have incorrectly given a C at their position −3650 in the sequence of Quattrochi & Tukey (1989) when this should be a T.] Of note, Nakajima and colleagues also report a C_{-3484} deletion; we did not find this deletion in any of our samples sequenced. It is therefore possible that this

represents a further polymorphic site, which is present in Japanese.

Three polymorphisms in *CYP1A2* which appear to be associated with a functional change have been recently reported: a $C_{-164}A$ substitution in intron 1 of Caucasians [allele *CYP1A2*1F*, Macleod *et al.* (1998); Sachse *et al.* (1999)], a $C_{63}G$ substitution in exon 2 of Han Taiwanese [allele *CYP1A2*2*, Huang *et al.* (1999)], and a $G_{-3858}A$ substitution in Japanese [allele *CYP1A2*1C*, Nakajima *et al.* (1999)]. However, the *CYP1A2*1F* allele was only associated with a slightly higher inducibility in smokers when present in the homozygous state; the *CYP1A2*2* substitution was very rare in the population studied and its functional consequence was not investigated; and the *CYP1A2*1C* substitution only appeared to be associated with a reduction in CYP1A2 activity in smokers. In addition, a recent report (Chida *et al.*, 1999) has described a T_{-2464} deletion (allele *CYP1A2*1D*, frequency 0.42) and a $T_{-740}G$ substitution in Japanese (allele *CYP1A2*1E*, frequency 0.082). The functional significance of these latter allelic variants has not been described. CYP1A2 phenotyping studies have varied in their conclusions as to whether the distributions support the existence of an underlying genetic component to CYP1A2 variability and, although mathematical analysis of phenotyping methodology (Rostami-Hodjegan *et al.*, 1996) indicates that the ratio used by Kalow and Tang (1991a,b) should be the most discriminant of bimodality and independent of the renal clearance of caffeine and paraxanthine, experimentally, only skewed unimodal or log normal distributions of this ratio have been observed (Kalow & Tang, 1991a,b; Vistisen *et al.*, 1992; Tang *et al.*, 1994). This may be because these studies have been performed mainly in Caucasians, and other work (Relling *et al.*, 1992; Chang *et al.*, 1997) indicates that there may be a higher frequency of CYP1A2 poor metabolizers or a lower population mean CYP1A2 activity in other ethnic groups. Studies which have supported a bimodal or trimodal population distribution of CYP1A2 activity (Devonshire *et al.*, 1983; Butler *et al.*, 1992; Nakajima *et al.*, 1994) report a frequency of poor metabolizers of 10–14%, in both smokers and non-smokers. The *CYP1A2* polymorphisms reported to date are not sufficient to account for this apparent frequency of poor metabolizers.

In a recent CYP1A2 phenotyping study (Tantcheva-Poór *et al.*, 1999), using the salivary paraxanthine to caffeine ratio (which correlates highly with systemic caffeine clearance) in 786 Caucasians, the maximum likelihood test showed that the overall distribution of residuals was best described by the sum of two separate normal distributions, with 52% of subjects lying within the first normal distribution. Nonetheless, this apparent polymorphism could be equally well explained by non-specific deviation from the normal distribution, as evidenced by minor skew seen in the *P–P* plot. Indeed, as the bimodal interpretation gives a poor metabolizer frequency substantially higher than that reported by earlier authors (Devonshire *et al.*, 1983; Butler *et al.*, 1992; Nakajima *et al.*, 1994), we conclude that this study does not support the existence of polymorphism in *CYP1A2* in the studied population.

As well as known environmental influences on CYP1A2 activity and intrinsic genetic variation, there are other possible causes of variation in CYP1A2 activity, such as gene–gene interactions. Coordinate regulation of *CYP1A2* and the UDP-glucuronyltransferase *UGT1.6* has been reported (Bock *et al.*, 1994). Macleod *et al.* (1997) reported that individuals possessing the glutathione transferase *GSTM1*0* allele had higher CYP1A2 activity for both non-induced and induced conditions, while individuals having the Ile/Ile *CYP1A1* genotype had higher CYP1A2 activity in the non-induced state, but lower CYP1A2 activity in the induced state than those with the Ile/Val genotype. In their studies of the colon carcinoma cell line LS180, Li *et al.* (1998) found that the concentration–response curves for induction by TCDD or 3-methylcholanthrene and the time courses of induction were very similar for CYP1A1, CYP1A2, and CYP1B1 [another enzyme of the CYP1 family; Shimada *et al.* (1997)], implying that the regulation of these enzymes may occur via a common pathway. Indeed, each member of the CYP1 family is inducible by TCDD via the arylhydrocarbon receptor (AhR) mechanism (Rowlands & Gustafsson, 1997). In mice, the genetic difference in susceptibility of different strains to TCDD-induced toxicity has been shown to correlate with polymorphism of the AhR (Nebert, 1989; Chang *et al.*, 1993). Polymorphisms in the human AhR have been identified (Garte & Sogawa, 1999; Nebert *et al.*, 1999), and it is possible that these may affect the inducibility of the CYP1 enzymes in man.

There are deficiencies in the studies conducted to date that have searched for functional polymorphisms in *CYP1A2*. Nakajima *et al.* (1994) sequenced the seven exons, exon–intron junctions, and the 5′ flanking region to –3470 in only five individuals (of different phenotypes); in their later paper, Nakajima *et al.* (1999) sequenced –3418 to –4065 in the same five individuals; Welfare *et al.* (1999) performed screening by SSCP of the seven exons, exon–intron boundaries, and two upstream regions in only 19 individuals (of different phenotypes), and sequenced

only two; Huang *et al.* (1999) sequenced the exons of only eight Taiwanese; Sachse *et al.* (1999) only investigated the intron 1 polymorphism; and our screening focused on an upstream region, sequencing 20 Caucasians, 31 Taiwanese, and 10 African Americans. We conclude that, first, further phenotyping work is warranted in this field, e.g. use of the Kalow and Tang (1991a) index of CYP1A2 activity in several different ethnic groups, carefully controlling for all possible confounding variables and, second, this should be combined with screening for functional polymorphisms in *CYP1A2* using reliable methods such as PCR-sequencing in a larger number of subjects than has been screened by this method to date (e.g. 50 from three different ethnic groups), and covering regions of *CYP1A2* that have not been screened. Finally, characterization of the positions of *CYP1A1* and *CYP1A2* on chromosome 15 and investigation of their possible coregulation, as well as the putative coregulation of *CYP1A2* with genes such as *CYP1B1*, the *UGT*s, and *GST*s, should prove fruitful.

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Clozapine pharmacokinetics and pharmacodynamics studied with CYP1A2-null mice

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The aim of this study was to use the CYP1A2-null mouse to investigate the in-vivo contribution of CYP1A2 to clozapine pharmacokinetics and pharmacodynamics. An intraperitoneal injection of 10 mg/kg clozapine was administered to four male CYP1A2 -/- mice and four male wild-type mice. Clozapine, desmethylclozapine, and clozapine *N*-oxide concentrations in sequential tail blood samples were measured by HPLC with UV detection. Behavioural parameters were recorded at each time point. The area under the curve (AUC) of clozapine was 2.6 times greater, the clearance of clozapine was 2.6 times slower, and the half-life was 1.2 times longer in the CYP1A2 -/- mice ($p = 0.0143$) as compared to the wild-type mice. Sixty-one percent of the clozapine clearance in wild-type mice was calculated to be mediated by CYP1A2. The AUC of desmethylclozapine was 1.6 times lower in the CYP1A2 -/- mice compared to the wild-type mice ($p = 0.0286$), while there was a trend for the AUC of clozapine *N*-oxide to be greater in the CYP1A2 -/- mice ($p = 0.0571$). The CYP1A2 -/- mice were significantly more drowsy and showed more motor impairment ($p = 0.0145$) and myoclonus than the wild-type mice. Our results indicate that, *in vivo*, CYP1A2 is the major determinant of clozapine clearance, contributes significantly to the demethylation of clozapine, and has a negligible contribution to the *N*-oxidation. Our data also indicate that CYP1A2 poor metabolizers might be more susceptible than extensive metabolizers to dose-related adverse effects of clozapine, such as sedation, myoclonus and seizures.

Key words: clozapine; CYP1A2; cytochrome P450; knockout; metabolism; pharmacodynamics; pharmacokinetics

Introduction

Clozapine is an atypical antipsychotic that is employed in the UK in cases of treatment-resistant schizophrenia or schizoaffective disorder. It is effective in approximately 30–60% of patients unresponsive to typical antipsychotics (Kane *et al.*, 1988; Kane, 1992), but the risk of agranulocytosis (0.7% in the first year of treatment, Atkin *et al.*, 1996) means that regular haematological monitoring is necessary and limits its use to treatment-resistant cases.

There is wide interindividual variation (approximately 10- to 50-fold) in the plasma levels of clozapine for a given dose (Perry *et al.*, 1991; Potkin *et al.*, 1994; Olesen *et al.*, 1995) and several studies indicate that clozapine concentrations of at least 350–420 μg/l are associated with clinical response (Perry *et al.*, 1991; Hasegawa *et al.*, 1993; Miller *et al.*, 1994; Potkin *et al.*, 1994; Kronig *et al.*, 1995). Clozapine is metabolized mainly in the liver, with the major metabolites being desmethylclozapine (or norclozapine) and clozapine *N*-oxide (Jann *et al.*, 1993). There have been several in-vitro studies investigating the roles of the hepatic cytochromes in the generation of these metabolites. Eiermann *et al.* (1997) concluded that CYP1A2 and CYP3A4 were both involved in the demethylation of clozapine, and CYP3A4 in the *N*-oxidation. However, Linnet and Olesen (1997) suggested that CYP2C19 and CYP3A4 would each mediate approximately 35% of clozapine metabolism at therapeutic concentrations, with CYP1A2 mediating only approximately 10%. Tugnait *et al.* (1999) found that CYP1A2 and CYP3A4 both catalyse the demethylation and *N*-oxidation of clozapine, but that CYP1A2 played a more important role in the former and CYP3A4 in the latter.

CYP1A2 and CYP3A4 both show wide interindividual variation in activity (Aitchison *et al.*, 2000a). Jerling *et al.* (1997) studied the population pharmacokinetics of clozapine and found that the clearance of clozapine was distributed in a similar way to indices of CYP1A2 activity, indicating that CYP1A2 could be the major determinant of clozapine clearance. Most studies have demonstrated a trimodal or bimodal distribution to the pattern of CYP1A2 activity in a population (Butler *et al.*, 1992; Lang *et al.*, 1994; Nakajima *et al.*, 1994; Schrenk *et al.*, 1998; Tantcheva-Poór *et al.*, 1999), with 12–14% of the population being CYP1A2 poor metabolizers.

Human and mouse CYP1A2 resemble each other closely in cDNA derived amino acid sequence (Kimura *et al.*, 1984; Jaiswal *et al.*, 1987) and in catalytic activity (Aoyama *et al.*, 1989). Given the inconsistencies in the conclusions of the above studies regarding the contribution of CYP1A2 to clozapine metabolism, we aimed to use the CYP1A2 –/– (null) mouse in order to investigate the in-vivo contribution of CYP1A2 to clozapine pharmacokinetics. In addition, we used the CYP1A2 –/– mouse as a model for CYP1A2 poor metabolizers, through the use of behavioural ratings aiming to draw conclusions regarding the pharmacodynamic effects of clozapine in CYP1A2 poor metabolizers.

Materials and methods

Materials

Clozapine was purchased from Sigma Chemicals Co. (St Louis, MO, USA), and desmethylclozapine and clozapine *N*-oxide from Research Biochemicals International (Natick, MA, USA). All other chemicals were of analytical-grade, available from commercial sources.

Animals

A line of CYP1A2 –/– mice were produced as described (Pineau *et al.*, 1995; Buters *et al.*, 1996). The wild-type mice were of the same (129/SV) genetic background. These two strains have been previously characterized in detail (Buters *et al.*, 1996). All mice were kept in a barrier facility with 2 p.p.m. chlorinated water, autoclaved food, and bedding under a 12 h light–dark cycle, 40% humidity, with free access to food and water. The animals were fasted for 12 h prior to dosing and for the duration of the experiment, but allowed water *ad libitum* throughout. Four male CYP1A2 –/– and four male wild-type animals were used. All mice had been born 81 days prior to the day of the pharmacokinetic study. The study protocol was approved by the Animal Care and Use Committee of the National Cancer Institute (National Institutes of Health, Bethesda, MD, USA).

Study design

A clozapine solution of 1 mg/ml was made (clozapine powder was dissolved in 0.1 M HCl, neutralized to pH 5–6 with 1 M NaOH, and made up to 1 mg/ml with deionized distilled water). All mice were weighed, and at time = 0, a dose of 10 mg/kg clozapine was given intraperitoneally. Eight sequential blood samples (each approximately 50 µl) per mouse were then taken by tail blood sampling at 5, 15, 30, 60, 120, 240, 360, and 480 min post injection (the injection times of the mice were approximately 20 min apart for ease of sampling). The method of tail blood sampling was by sequential clipping of the tail (2–3 mm of tail being cut at each interval), followed by cauterization to stop bleeding. The blood was collected in heparinized tubes (Sarstedt Ltd, Leicester, UK), and stored at –80 °C until analysis. Behavioural parameters (degree of drowsiness, motor incoordination) were recorded at each time point. After the last sample had been taken, the mice were killed by carbon dioxide asphyxiation.

HPLC analysis

Clozapine, desmethylclozapine, and clozapine *N*-oxide in the blood samples were analysed by HPLC with UV detection at 254 nm as described (Volpicelli *et al.*, 1993). The recovery from control rat serum at 500 ng/ml of clozapine, 500 ng/ml of desmethylclozapine, and 100 ng/ml of clozapine *N*-oxide was 78%, 84%, and 62%, respectively (n = 6 samples). A standard assay curve was completed and repeated six times using seven data points for clozapine of known concentrations in the range 10 ng/ml to 1000 ng/ml (r^2 = 0.996). The lower limit of detection was 5.0 ng/ml for clozapine, desmethylclozapine, and clozapine *N*-oxide, and the interassay and intra-assay coefficients of variation (CV) were 8.2% and 5.0%, respectively, for all three substances.

Pharmacokinetic data were calculated using the equation: Cl = D × F/AUC in which Cl is whole blood clearance, D is doses of clozapine, AUC is the area under the curve, and F is the fraction absorbed or bioavailability (model independent pharmacokinetics). The AUC was calculated using the linear trapezoidal rule using C (concentration) = 0 and t (time) = 0 and was extrapolated to infinity using Axum 5.0 (MathSoft, Inc, Cambridge, MA, USA). The half-life of clozapine in blood was calculated by linear regression of the ln transformed blood concentrations. The percentage of the clozapine clearance mediated by CYP1A2 ($\%Cl_{CYP1A2}$) in wild-type mice was estimated by the following relationship:

$$\%Cl_{CYP1A2} = (Cl_{wt} - Cl_{-/-})/Cl_{wt}$$

in which Cl_{wt} is the wild-type clozapine clearance and $Cl_{-/-}$ is the clozapine clearance in the CYP1A2 –/– mice. This simple relationship was used as it had already been established that the CYP1A2 –/– and wild-type mice lines did not differ from each other in parameters (such as liver function) that could affect the pharmacokinetics of clozapine (Buters *et al.*, 1996).

Behavioural effects ratings

In pilot studies, it was noted that clozapine at the dose administered caused drowsiness and motor impairment in the mice. A scale for the rating of these effects was therefore devised (Table 1). Mice were scored at each time point, just before tail blood sampling.

Statistical analysis

Statistical analyses were conducted using SPSS version 8.0 for Windows (SPSS, Chicago, IL, USA).

Table 1 Scale for rating behavioural effects of clozapine in mice

Clozapine behavioural effects ratings in mice	
Drowsiness	
0	Fully alert
1	Eyes half closed at rest
2	Eyes closed at rest, but easily rousable
3	Eyes closed at rest, difficult to rouse
Motor Impairment	
0	Normal posture at rest, move normally in cage on handling
1	Mild splaying of legs at rest, movements slowed and jerky, mild reduction in struggle on handling
2	Moderate splaying of legs at rest, little movement in cage, moderate reduction in struggle on handling
3	Prominent splaying of legs at rest (sprawled), no movement in cage, minimal struggle on handling

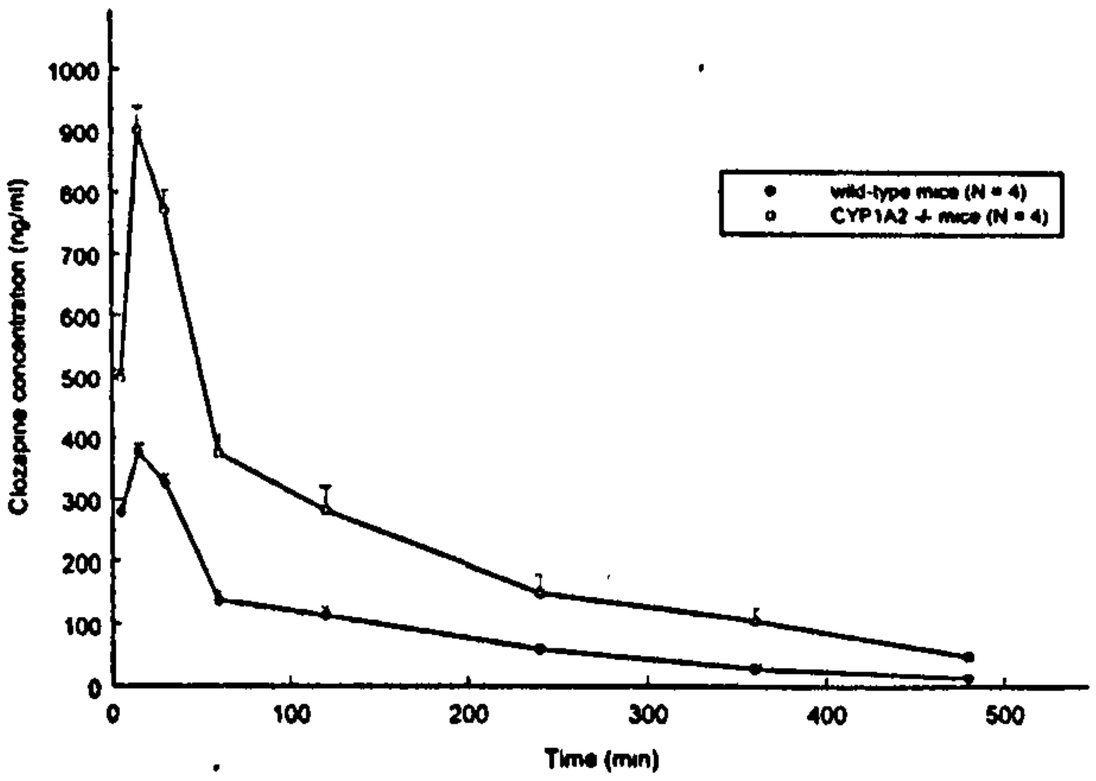

Figure 1 Whole blood clozapine concentration-time curves after a 10 mg/kg intraperitoneal dose of clozapine to male wild-type and CYP1A2 –/– mice, administered at time 0. Mean values ± SD are given

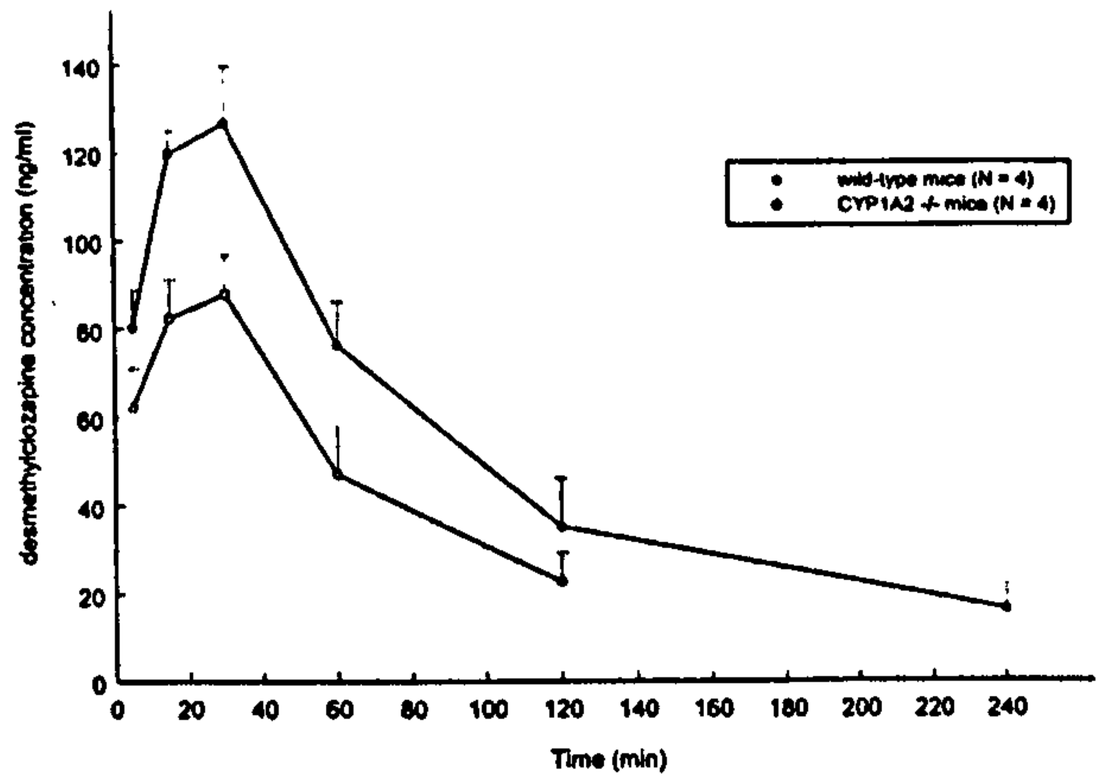

Figure 2 Whole blood desmethylclozapine concentration-time curves after the 10 mg/kg intraperitoneal dose of clozapine. For the wild-type mice at 240 min, the mean of two values is reported (the other two values were below the limit of detection, i.e. less than 5.0 ng/ml), otherwise the data are mean of four values ± SDs

Results

The mean weights (with standard deviations) of the wild-type and CYP1A2 –/– mice were 23.28 g (3.21) and 27.06 g (3.27), respectively. The Mann–Whitney *U*-test revealed a one-tailed *p*-value of 0.17, i.e. the two groups did not differ significantly in their mean weights. (Although mice weight and all the other parameters measured would be expected to show a normal distribution in the two groups, because there are only four mice per group, we consider that nonparametric testing is more appropriate and more stringent than parametric in this instance, and have hence used nonparametric testing throughout our analyses.)

Table 2 gives the pharmacokinetic parameters of clozapine, desmethylclozapine, and clozapine *N*-oxide in the two groups of mice, and Figs 1–3 show the concentrations of clozapine, desmethylclozapine, and clozapine *N*-oxide versus time. The AUC of clozapine was 2.6 times greater, the clearance of clozapine was 2.6 times slower, and the half-life was 1.2 times longer in the

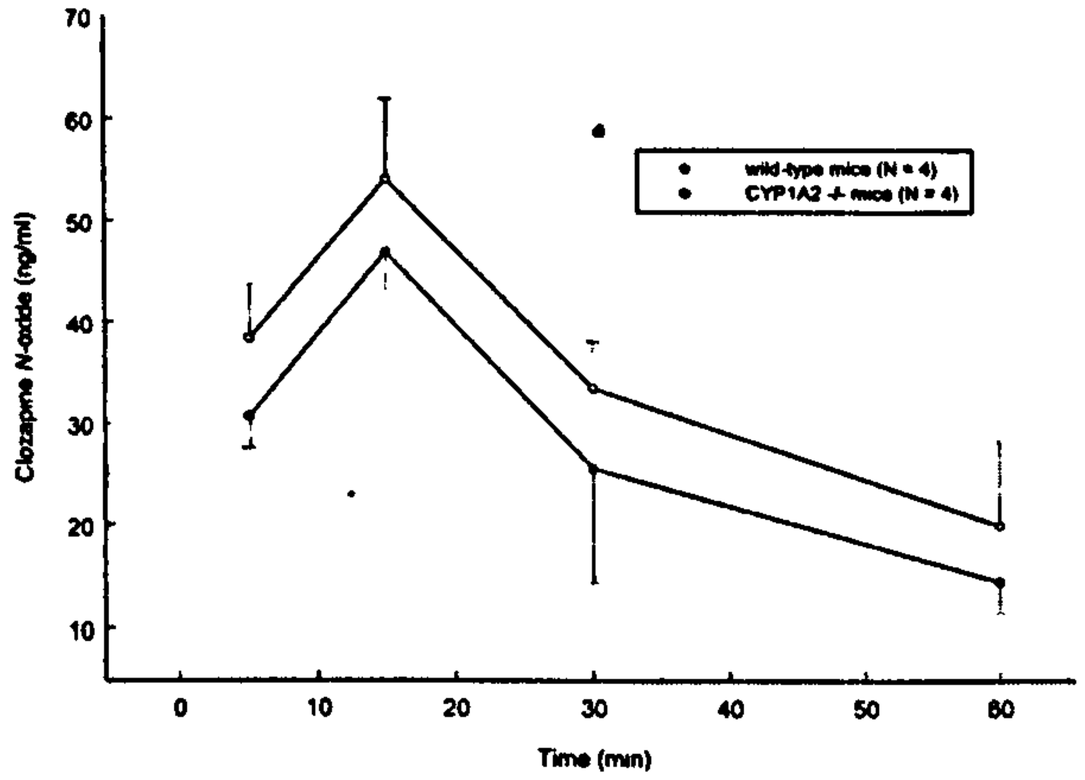

Figure 3 Whole blood clozapine *N*-oxide concentration-time curves (mean ± SD) after the 10 mg/kg intraperitoneal dose of clozapine. Values beyond $t = 60$ min for both wild-type and CYPA2 –/– mice were below the limit of detection (5.0 ng/ml)

Table 2 Pharmacokinetic parameters of clozapine, desmethylclozapine, and clozapine *N*-oxide in male wild-type and CYP1A–/– mice after a single 10 mg/kg intraperitoneal dose of clozapine (mean ± SD)

	Wild-type ($n = 4$)	CYP1A2 –/– ($n = 4$)
Clozapine $AUC_{0\rightarrow\infty}$ (mg/min/ml)	42.9 ± 3.9	111.3 ± 11.1**
Clozapine clearance/F (ml/kg/min)	0.234 ± 0.020	0.091 ± 0.009**
Clozapine half-life (min)	110.1 ± 8.1	129.8 ± 3.1**
Desmethylclozapine $AUC_{0\rightarrow\infty}$ (mg/min/ml)	11.9 ± 2.7	7.6 ± 2.2*
Clozapine N-oxide $AUC_{0\rightarrow\infty}$ (mg/min/ml)	2.0 ± 0.2	2.7 ± 0.8

*One-tailed $P = 0.0286$, ** one-tailed $P = 0.0143$ (Mann-Whitney U-test).

CYP1A2 –/– mice as compared to the wild-type mice. Sixty-one percent of the clozapine clearance in wild-type mice is mediated by CYP1A2. For desmethylclozapine, the AUC was 1.6 times lower in the CYP1A2 –/– mice as compared to the wild-type mice, while for clozapine *N*-oxide, although the AUC was 1.4 times greater in the CYP1A2 –/– mice, this difference did not quite reach statistical significance ($p = 0.0571$).

The behavioural effects of clozapine on the wild-type and CYP1A2 –/– mice are shown in Figs 4 and 5. The CYP1A2 –/– mice were significantly more drowsy and showed more motor impairment than the wild-type mice ($p = 0.0145$ in both instances, Mann–Whitney *U*-test). In addition, myoclonus was noted in one wild-type mouse at $t = 240$ min, in two CYP1A2 –/– mice at $t = 60$ min, in one CYP1A2 –/– mouse at $t = 120$ min, and in one CYP1A2 –/– mouse at $t = 120$ min and at $t = 240$ min.

Discussion

Our results indicate CYP1A2 contributes significantly to the demethylation of clozapine *in vivo*. The finding of a slightly greater AUC for clozapine *N*-oxide in the CYP1A2 –/– mice may be explained by the following: in the absence of CYP1A2, with a consequent higher concentration of clozapine, more clozapine was available to undergo *N*-oxidation by enzymes other than CYP1A2. If CYP1A2 contributes to the *N*-oxidation of clozapine in mice, it

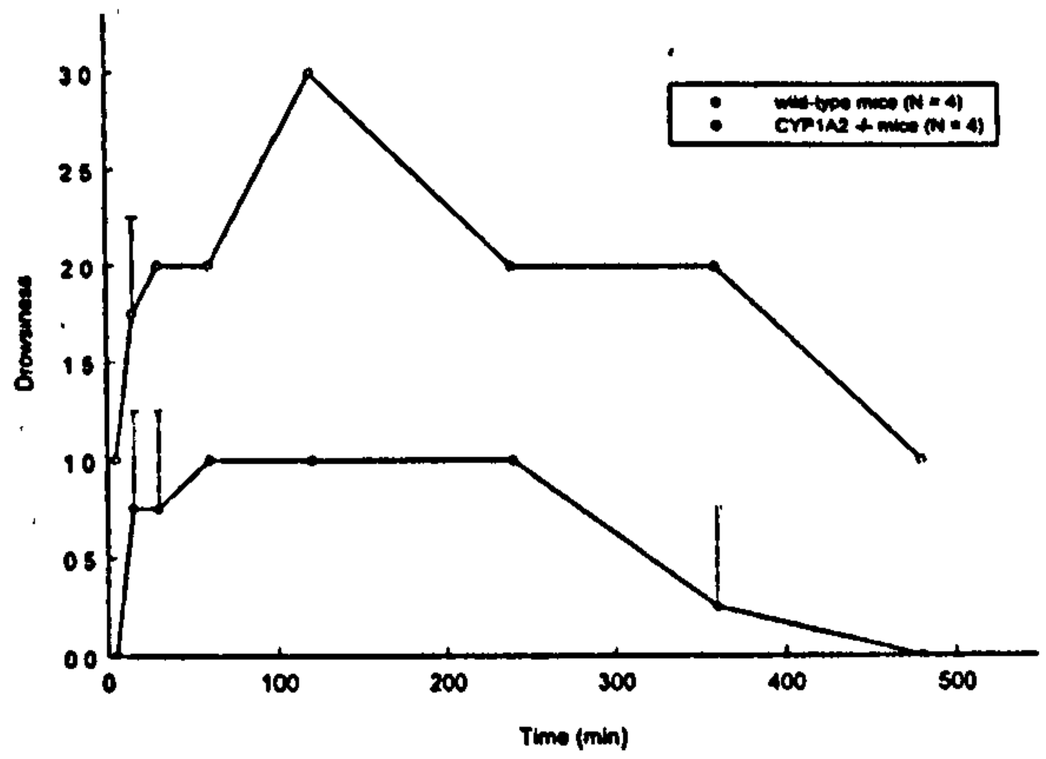

Figure 4 Degree of drowsiness versus time after the 10 mg/kg intraperitoneal dose of clozapine. Mice were rated for drowsiness using the criteria given in Table 1. Mean scores ± SD are given

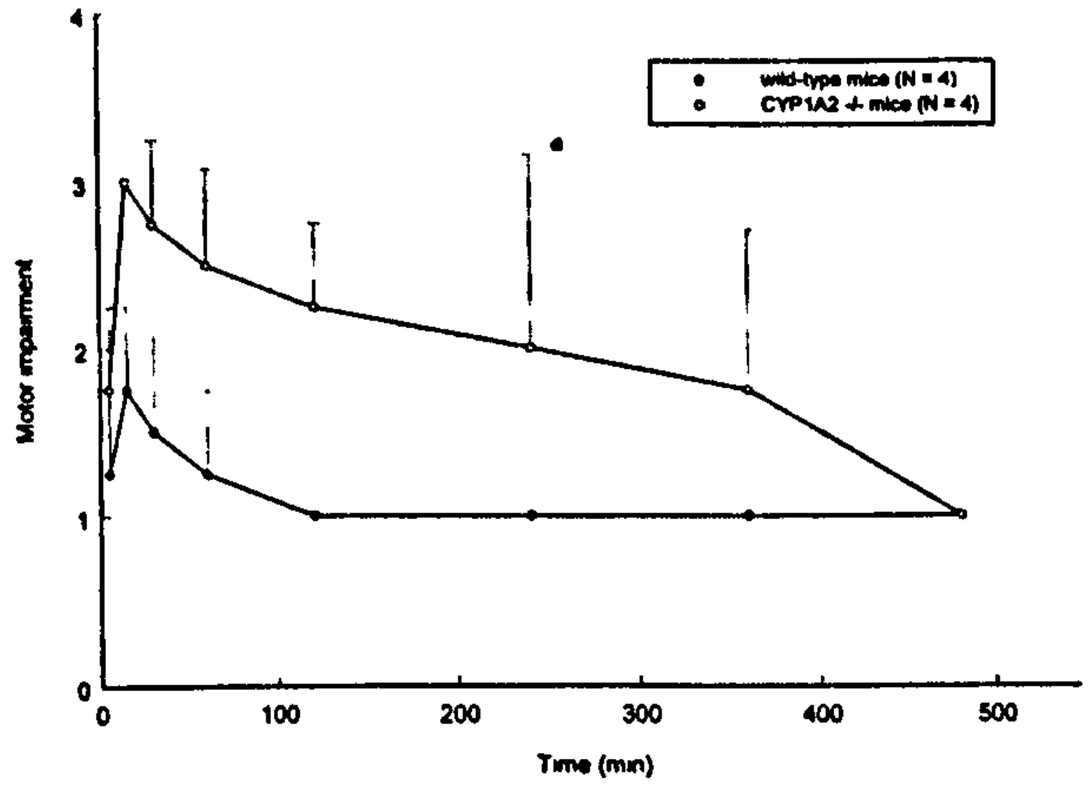

Figure 5 Degree of motor impairment vs. time after the 10 mg/kg intraperitoneal dose of clozapine. Motor impairment in the mice was rated using the criteria given in Table 1. Mean scores ± SD are given

must do so only to a very minor degree. These findings confirm the *in vitro* results of Eiermann *et al.* (1997) and are consistent with those of Tugnait *et al.* (1999). In addition, we have estimated that in wild-type mice, 61% of the clearance of clozapine is mediated by CYP1A2. This result is contrary to the estimation provided by Linnet and Olesen (1997), but in accordance with the conclusion of Jerling *et al.* (1997) in man. Our data can be extrapolated to man because CYP1A2 expression and catalytic activities are conserved between mice and humans (Kimura *et al.*, 1984; Jaiswal *et al.*, 1987; Aoyama *et al.*, 1989).

The behavioural data show that after clozapine adminstration, the CYP1A2-null mice were significantly more drowsy, showed more motor impairment, and had more myoclonus than the wild-type mice. Clinical data indicate that sedation is the most commonly reported adverse effect of clozapine, occurring in 39% of patients (Safferman *et al.*, 1991). Our data indicate that for a given dose of clozapine, CYP1A2 poor metabolizers might show a greater degree of sedation than extensive metabolizers. Myoclonus occurs in 2% of patients on clozapine (Lieberman and Safferman, 1992) and may progress to generalized seizure activity (Berman *et al.*, 1992; Gouzoulas *et al.*, 1993; Meltzer and Ranjan, 1994). Seizures are a dose-related adverse effect, the frequency of seizures at clozapine doses of less than 300 mg/day, 300–600 mg/day, and above 600 mg/day being 1%, 2.7%, and 4.4%, respectively (Devinsky *et al.*, 1991). Our data similarly indicate that CYP1A2 poor metabolizers might be more prone to myoclonus, and therefore might be at greater risk of generalized seizure activity for a given dose of clozapine. The risk of this and other dose-related adverse effects could be minimized by titrating the dose up more slowly than usual, monitoring carefully for the emergence of effects such as sedation. The fact that the CYP1A2 –/– mice had significantly higher levels of clozapine would also imply that CYP1A2 poor metabolizers would respond at lower doses of clozapine than extensive metabolizers. The dose of clozapine employed in this study (10 mg/kg) was chosen as the results of preliminary experiments indicated that this dose was necessary to achieve informative data for desmethylclozapine and clozapine *N*-oxide, and would be equivalent to a dose of approximately 700 mg or 600 mg clozapine in an average man or woman, respectively. In a study of 12 760 clozapine recipients in the UK and Ireland, the mean clozapine dose after 12 weeks of treatment was 388 mg/day, the mean maximum dose was 462 mg/day, and 41% had a peak dose of more than 500 mg/day (Munro *et al.*, 1999). Our study indicates that daily doses of greater than 600–700 mg (especially as a single dose) should not be used in CYP1A2 poor metabolizers.

Parallels may be drawn between our study and studies investigating the correlation between adverse effects of typical antipsychotics and CYP2D6 metabolizer status. In a single dose pharmacokinetic study of perphenazine administered to six CYP2D6 poor metabolizers and six CYP2D6 extensive metabolizers, the poor metabolizers reported more adverse effects, especially tiredness (Dahl-Puustinen *et al.*, 1989). Similarly, in a study investigating severe adverse effects during the first days of treatment with a phenothiazine or haloperidol, CYP2D6 poor metabolizers had a higher incidence of concentration-dependent effects, such as oversedation, postural hypotension and autonomic effects (Spina *et al.*, 1992). However, concentration-dependent effects tend to reduce with time (Aitchison *et al.*, 1999); in the case of clozapine, patients usually develop tolerance to the sedative effects within 4–6 weeks of treatment (Marinkovic *et al.*, 1994). Correlations between metabolizer status and concentration-dependent effects are therefore most relevant to the initial, dose-finding stages of prescribing.

Other adverse effects of clozapine, such as neutropenia and agranulocytosis, do not appear to be concentration-dependent. The peak incidence of neutropenia and agranulocytosis occurs within the first 6–18 weeks of clozapine treatment, being 1.27% and 0.7%, respectively, and the risk of both decreases with increases in clozapine dose (Munro *et al.*, 1999). The pathophysiological mechanism of agranulocyotosis is uncertain, but hypotheses include a direct toxic effect on neutrophils or haemopoietic precursors (Veys *et al.*, 1992; Gerson *et al.*, 1994; Deliliers *et al.*, 1998), and an immunological basis (Pisciottta *et al.*, 1992), the latter with a possible genetic predisposition (Lieberman *et al.*, 1990; Corzo *et al.*, 1995). Desmethylclozapine at high concentrations has been seen to have a toxic effect on the precursors of both myeloid and erythroid lineages (Gerson *et al.*, 1994), and the desmethylclozapine/clozapine ratio has been seen to be inversely correlated with neutrophil count in patients treated with clozapine (Mauri *et al.*, 1998). If the formation of

desmethylclozapine is primarily CYP1A2-dependent, then the desmethylclozapine/clozapine ratio should reflect CYP1A2 activity. This would imply that individuals with relatively high CYP1A2 activity could be at greater risk of clozapine-induced neutropenia and agranulocytosis. However, other studies have failed to find an association between desmethylclozapine levels or desmethylclozapine/clozapine ratio and granulocyte counts (Hasegawa *et al.*, 1994; Combs *et al.*, 1997).

Another hypothesis regarding the mechanism of agranulocytosis is that it involves metabolic activation of clozapine with the formation of free radicals which are able to bind covalently to neutrophil or bone marrow proteins, then leading to agranulocytosis either by a direct toxic (analagous to the covalent binding of acetaminophen in the liver, Pumford and Halmes, 1997) or an immunological route (Fischer *et al.*, 1991; Liu and Uetrecht, 1995; Maggs *et al.*, 1995; Pirmohamed *et al.*, 1995; Gardner *et al.*, 1998a.b). Chemically reactive metabolites of clozapine may be formed by neutrophil myeloperoxidase (Gardner *et al.*, 1998a) or by cytochrome P450s CYP3A, CYP2C9, and CYP2E1 (Pirmohamed *et al.*, 1995). The formation of the reactive metabolites catalysed by the P450s is reversible (Pirmohamed *et al.*, 1995); under conditions of relatively low CYP1A2 activity, the increase in clozapine concentration would be expected to lead to an increase in the proportion of clozapine passing down the pathway of reactive metabolite formation. If this hypothesis were correct, then CYP1A2 poor metabolizer status would be expected to be associated with agranulocytosis. However, agranulocytosis and CYP1A2 activity do not show the same ethnic stratification, i.e. agranulocytosis occurs at a greater frequency in Asians but not Orientals or African-Caribbeans compared to Caucasians (Munro *et al.*, 1999), while CYP1A2 activity appears to be lower in Orientals and Blacks (Relling *et al.*, 1992; Chang *et al.*, 1997; Le Marchand *et al.*, 1997). Nonetheless, it is possible that combined enzyme deficiency (e.g. CYP1A2 poor metabolizer status combined with a glutathione *S*-transferase deficiency) could further increase an individual's risk for the formation of potentially harmful reactive intermediates. This theory is supported by the analagous finding of Rojas *et al.* (1998) that smokers with combined CYP1A1 and glutathione *S*-transferase M1 (GSTM1) deficiency showed significantly higher levels of activated covalently bound metabolites than individuals with CYP1A1 or GSTM1 deficiency alone.

An allelic variant of *CYP1A2*, *CYP1A2*1C* (–3858G to A) occurring at an allele frequency of 0.23 in Japanese (Nakajima *et al.*, 1999), which appears to be associated with reduced CYP1A2 activity in smokers, has recently been identified. Other allelic variants, whose functional consequences have not been described, have also been reported: *CYP1A2*1D* (–2464delT, frequency 0.42 in Japanese, Chida *et al.*, 1999), *CYP1A2*1E* (–740T → G, frequency 0.082 in Japanese, Chida *et al.*, 1999), and *CYP1A2*2* (63C → G, frequency < 0.01 in Chinese, Huang *et al.*, 1999). In addition, three further polymorphisms in the *CYP1A2* 5′ flanking region have been identified (Aitchison *et al.*, 2000b). Although the alleles identified to date would appear not to account for the bi- or trimodality of CYP1A2 activity in a population, it is likely that in the near future the *CYP1A2* alleles responsible for the poor or rapid metabolizer phenotypes will be identified. Pre-prescribing genotyping for CYP1A2 activity would then become feasible, and could lead to the judicious use of particularly low doses of clozapine and other drugs that are metabolized by CYP1A2. Furthermore, in combination with assays for polymorphisms in other drug metabolizing enzymes such as the glutathione transferases, genotyping for CYP1A2 activity could yield useful information regarding genetic susceptibility to idiosyncratic drug reactions such as agranulocytosis. In the mean time, since it is known that CYP1A2 activity is lower in Orientals and Blacks (Aitchison *et al.*, 2000a), we would suggest that in these groups it would be wise to commence with particularly low doses of clozapine and other CYP1A2-metabolized drugs, and to use a slow dose escalation procedure, monitoring carefully for the emergence of concentration-dependent adverse effects such as sedation and myoclonus.

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THE RELEVANCE OF ETHNIC INFLUENCES ON PHARMACOGENETICS TO THE TREATMENT OF PSYCHOSIS

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CONTENTS

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SUMMARY

Interethnic variation amongst the drug metabolising enzymes relevant to the treatment of psychosis is reviewed. The frequency of genetically determined variants at the extremes of enzyme activity is seen to vary considerably between different ethnic groups; in addition, a shift in the frequency distribution giving an overall lower population mean activity may occur. The role of dietary and other environmental influences in the generation of interethnic variation in cytochrome activity is also discussed. Clinical studies pertinent to this variation are reviewed. It is suggested that the reason for conflicting data from some clinical studies is the existence of overlapping substrate specificity, so that one cytochrome is able to substitute for another. Individuals deficient for more than one cytochrome would be likely to show much more pronounced clinical effects than those showing single cytochrome deficiency.

KEY WORDS

ethnicity, pharmacogenetics, metabolism, cytochrome P450, antipsychotics

INTRODUCTION

The term pharmacogenetics was coined when adverse drug reactions were first attributed to genetic factors /1/. Genetic factors affect both drug metabolism (pharmacokinetics) and drug response at the level of the target organ (pharmacodynamics). With respect to interethnic variation affecting the treatment of psychosis, pharmacokinetic genetic factors, encoding the drug metabolising enzymes (or DMEs), have been far more extensively investigated than interethnic variation affecting pharmacodynamic genetic factors. This review will therefore focus mainly on the DMEs.

An important subset of the DMEs is the group of cytochrome P450 enzymes (CYPs), or haem-thiolate proteins. These enzymes metabolise not only drugs, but also endogenous compounds (*e.g.* steroids), plant products, and man-made environmental toxins. Three members of this family of enzymes have been described to be involved in the metabolism of antipsychotics: CYP2D6, CYP3A4, and CYP1A2.

Other enzymes in this family, including members of the CYP2C subfamily, should also be considered, especially with regard to drug-drug interactions.

1. CYP2D6

1.1 Genetic variation in CYP2D6

Four different levels of activity of CYP2D6 have been identified, through the use of probe drugs which are metabolised by the enzyme. An individual may be termed an ultrarapid metaboliser (UM), extensive metaboliser (EM), intermediate metaboliser (IM), or poor metaboliser (PM). This variation in enzymatic activity is due to multiple allelic variants of *CYP2D6* (the gene encoding the protein), the frequencies of which differ in different ethnic groups /2,3/.

In Caucasian populations, the frequency of PMs is 5-10% /4/, while in Black Africans the frequency is 0-8% /5-7/, in African-Americans the frequency is 3.7% /8/, and in Orientals (Chinese, Japanese, and Koreans having been studied) the frequency is approximately 1% /9-14/. In addition, a lower population mean enzyme activity has been observed in Chinese, Zimbabweans, and Ghanaians as compared to Caucasians. The low PM frequency in Orientals is caused mainly by the very low frequency of the *CYP2D6*4* mutant allele, an allele which is associated with absent enzyme activity and accounts for about 66% of PM alleles in Caucasians /3/. The lower population mean enzyme activity has been attributed to the relatively high frequency of *CYP2D6*10* in the Chinese, and of *CYP2D6*17* in the Ghanaians and Zimbabweans, both of which alleles being associated with diminished CYP2D6 activity /15-18/. The *CYP2D6*17* allele also occurs at a greater frequency in African-Americans /8/. Dahl *et al.* /19/ compared findings in a pilot study on Koreans, Chinese, and Japanese, and found that the frequency of *CYP2D6*10A* and *CYP2D6*10B* was somewhat lower among the Koreans than among the Chinese or Japanese. Therefore findings from one ethnic group may not be applicable to another geographically close and apparently similar ethnic group. Canadian Native Indians are descendants of North Asian populations, and have been found to resemble Chinese in terms of PM frequency, but to lack the shift towards a lower mean enzyme activity (Fig. 1) /20/. This was

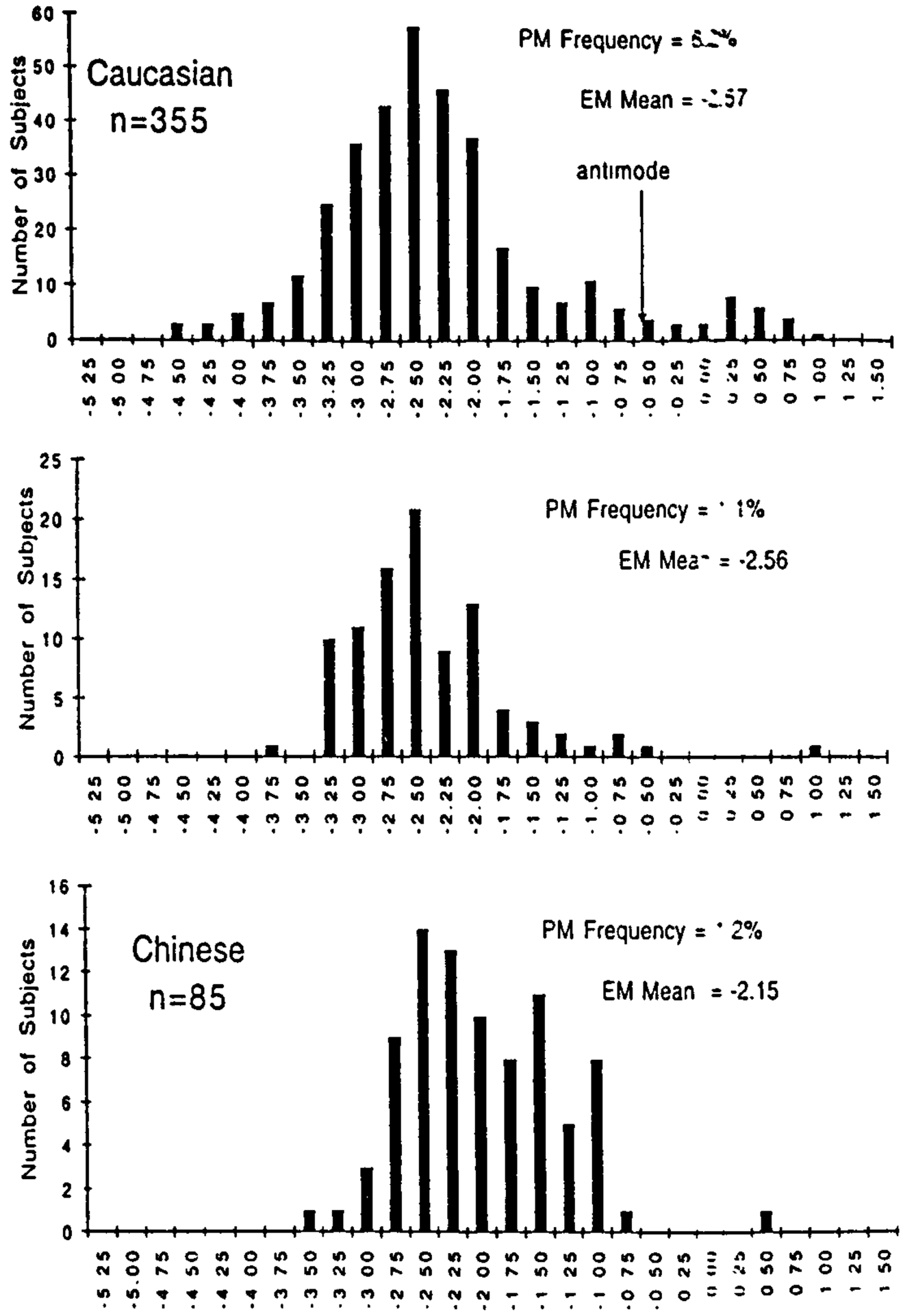

Fig. 1: Frequency distributions of dextromethorphan O-demethylation ratios in Caucasian, Canadian Native Indian (central graph, n=115), and Chinese populations. Dextromethorphan is metabolised by CYP2D6, and the O-demethylation ratio (ODMR) is a measure of CYP2D6 enzyme activity, where the higher the ratio, the lower the enzyme activity. (From: Nowak MP, Tyndale RF, Sellers EM. CYP2D6 phenotype and genotype in a Canadian Native Indian population. Pharmacogenetics 1997; 7: 147, with permission.)

seen to be due to a lower frequency of the *CYP2D6*3* and *CYP2D6*4* mutant alleles relative to Caucasians, and a lower frequency of *CYP2D6*10* compared with the Chinese. Similarly, a relatively low frequency of *CYP2D6*10* has been found in the South-Amerindian population of Chile /21/. This genetic drift has been interpreted as possibly due to a founder effect, mitochondrial DNA sequence variations revealing that these two groups of Amerindians were derived from a small number of maternal lineages /22/, or due to genetic selection pressures by dietary or other environmental factors. Middle Eastern populations show a very low frequency of CYP2D6 PMs, resembling Orientals rather than Caucasians in phenotyping studies (reviewed in /23/).

A further factor to be considered amongst individuals of lower CYP2D6 activity is that in Black African populations, individuals who appear to be PMs when tested with one drug may not be PMs when tested with another drug, the two drugs both being metabolised similarly by CYP2D6 in Caucasians /5,24-26/. This has been suggested to be due either to the presence of an as yet unidentified CYP2D6 variant with differential substrate specificity, or to inter-ethnic variations in conjugation and/or renal tubular transport.

At the other end of the spectrum of enzyme activity, the frequency of UMs also differs markedly between different ethnic groups, being 0.8-2% in Danes or Swedes /27,28/, 3.6% in Germans /29/, less than 5% in Black Zimbabweans /7,30/, 7% in Spaniards /31/, 20% in Saudi Arabians /32/, and 29% in Ethiopians /33/.

1.2 Clinical relevance

It has been suggested that PMs and IMs might show a tendency towards higher serum levels of drugs metabolised by CYP2D6 for a given dose, and might therefore be more susceptible to adverse effects (*i.e.* be treatment-intolerant), whereas UMs might show particularly low serum levels at standard doses and might therefore appear to be treatment-refractory. In accordance with this, in studies on normal volunteers, PMs were shown to have significantly higher serum levels of perphenazine /34/ and zuclophenthixol /35/, while the oral clearance of perphenazine and zuclopenthixol in patients on continuous treatment was shown to be significantly predicted by CYP2D6 genotype /36/.

CYP2D6 is also involved in the metabolism of haloperidol, fluphenazine, and trifluperidol /37/. Case reports support an association between PM status and a higher susceptibility to adverse effects /38,39/. A trend towards an excess of mutant *CYP2D6* alleles has been seen in schizophrenics with movement disorders /40/. However, another study has not found an excess of PMs amongst schizophrenics intolerant of typical antipsychotics (Aitchison *et al.*, unpublished data).

Haloperidol concentrations have been found to be elevated in Chinese patients suffering from schizophrenia /41/. Although this could also be due to interethnic variations in CYP3A4 activity (see below), it would be consistent with the lower mean CYP2D6 activity seen in Chinese. Nyberg *et al.* /42/ showed that a PM of CYP2D6 had higher concentrations of plasma haloperidol throughout a 4-week treatment period with haloperidol decanoate compared with 7 EMs of CYP2D6. Suzuki *et al.* /43/ studied 50 Japanese schizophrenic patients, and found a higher mean steady-state plasma haloperidol concentration in patients with one mutant allele (mainly *CYP2D6*10*) as compared with patients with no mutant alleles, and a higher mean steady-state plasma reduced haloperidol concentration in patients with 1 or 2 mutant alleles as compared with patients with no mutant alleles. However, Lin *et al.* /44/ found that on a fixed dose weight-adjusted regime, Oriental patients had only a slightly increased mean haloperidol plasma level. Nonetheless, they had a significantly higher rating for extra pyramidal symptoms (EPS), and also higher concentrations of prolactin in response to haloperidol /45/. This could be due to a pharmacodynamic interethnic difference, *e.g.* due to variability in the dopamine D_2 receptor, or to interethnic differences in CYP3A4 (see below).

With regard to UM status, two patients have been described for whom particularly high doses of tricyclic antidepressants metabolised by CYP2D6 were required in order to achieve a therapeutic response /46/. However, in a study comparing 73 patients who were treated with typical antipsychotics and found not to be treatment-refractory with 235 treatment-refractory patients, an excess of UMs was not found in the refractory group /47/. On the contrary, a trend towards an excess of UMs was found in the non-refractory group, although the numbers of UMs were very low in both groups (2 and 3 in the refractory and non-refractory groups, respectively). This argues against

ultrarapid hydroxylation by CYP2D6 of typical antipsychotics being a major cause of failure to respond to treatment with these agents.

Risperidone and sertindole are metabolised by CYP2D6 to 9-OH-risperidone and dehydrosertindole respectively. Although 9-OH-risperidone has antipsychotic activity, and the *combined* plasma concentrations of risperidone and this metabolite would be expected to be similar for individuals with different ends of the spectrum of CYP2D6 activity, it differs somewhat from risperidone in its *in vitro* receptor profiles and protein- and brain-binding characteristics /48/. The antipsychotic activity of dehydrosertindole appears to be less than sertindole, with the mean serum levels of sertindole being up to 3-fold higher in PMs than in EMs. However, trials to date do not show a clear relationship between sertindole concentrations and therapeutic effect, and sertindole has a significant alternative pathway via CYP3A4. *In vitro* work reported a role for CYP2D6 in clozapine metabolism /49/; however, no association has been found between CYP2D6 genotype and clozapine response /50/. This is consistent with later work that has shown that the predominant enzymes in clozapine metabolism are CYP1A2 and CYP3A4 /51/. Nonetheless, the inhibitory effect of the selective serotonin reuptake inhibitors (SSRIs) paroxetine, fluoxetine, and sertraline on clozapine metabolism may be partly accounted for by CYP2D6-mediated interactions /52/.

In summary, the clinical data regarding CYP2D6 metaboliser status and response are confusing. It is possible that in certain individuals, CYP2D6 plays a factor in either the generation of adverse effects or lack of therapeutic response, but that when studies are conducted on patient populations, other factors, such as the overlapping substrate specificities of CYP enzymes, or pharmacodynamic factors cloud the picture so that the results seen in case reports are not replicated in larger studies.

2. CYP3A4

2.1 Variability in CYP3A4 activity

CYP3A4 is present in the liver and small intestine, and plays a role in the metabolism of many typical antipsychotics, sertindole, and clozapine /48/. This enyzme can be induced, inhibited, or inactivated

by drugs as well as environmental factors including food substances. Interpopulation variation in activity may therefore arise not only secondary to intrinsic variation in enzyme activity, but also secondary to the effect of environmental agents.

Nifedipine is a cardiovascular drug that is metabolised by CYP3A4 and has been used as a probe drug to investigate CYP3A4 activity in different populations. It has been shown that South Asians (from the Indian subcontinent) oxidise nifedipine at a significantly slower rate than Caucasians (Fig. 2) /53,54/, resulting in sustained haemodynamic changes. In the first study by Ahsan and colleagues, the South Asians had retained their original dietary practices, whereas the Caucasians consumed a typical Western diet. The effect of diet was studied in six Caucasians by giving them an Indian diet for 3 days prior to the administration of nifedipine; no significant difference in any of the pharmacokinetic parameters was detected.

Similarly, the N-demethylation of codeine, which is catalysed by an enzyme of the CYP3A subfamily, occurs at a significantly slower rate in Chinese as compared to Caucasians /55/. Interestingly, Chinese have also been shown to have a significantly lower mean codeine N-demethylation activity as compared to Japanese /56/.

Recently an A→G point mutation has been found in the nifedipine specific element (NFSE) at position -289 in the 5′ flanking region of the CYP3A4 gene /57/. This mutation has been further analysed in 59 Taiwanese, 59 Finnish, and 75 African-American subjects, and found to show an allelic frequency of 0%, 4.2%, and 66.7% in these populations, respectively (Sata, personal communication). Functional studies have not yet been performed, but it could well be the case that this mutation is the primary mutation responsible for interethnic variations in CYP3A4 activity.

2.2 Clinical considerations for CYP3A4

Drugs and food substances may act on the CYP3A4 present in the small intestine as well as that present in the liver. Indeed, the effect of a given agent on the two CYP3A4s may differ. For example, consumption of some furanocourmarins present in grapefruit juice can cause inactivation of enterocyte CYP3A4 while having no detectable effect on liver CYP3A4 activity /58/. Conversely, some oral drug regimens have been shown to increase liver CYP3A4 activity while having no effect on small bowel CYP3A4. It appears that some drugs

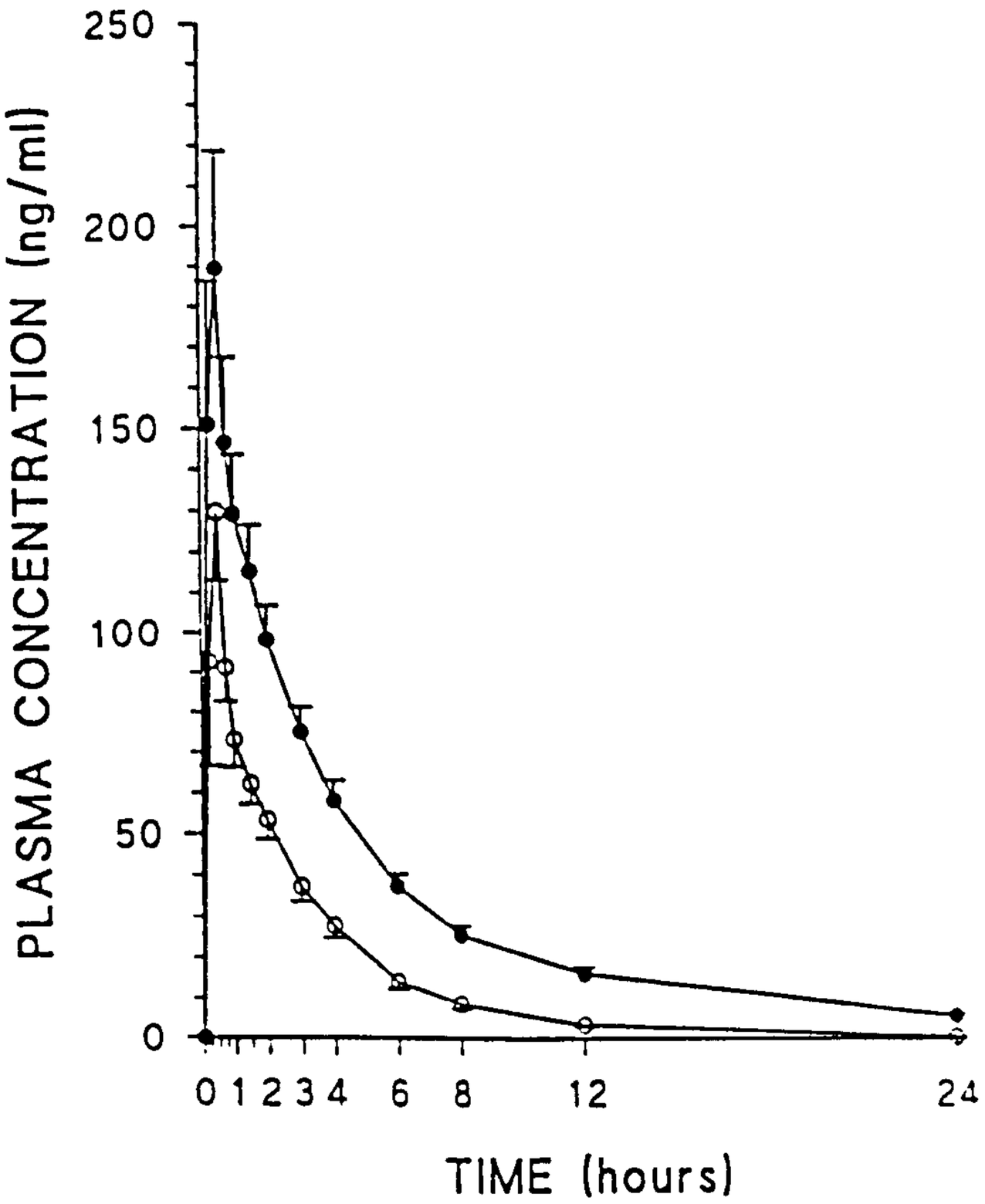

Fig. 2: Plasma concentration-time curves for nifedipine after 20 mg capsules were administered to Caucasian subjects (open circles; n=27) and South Asians (solid circles; n=30). Data are mean values with standard errors shown as vertical bars. (From: Ahsan CH, Renwick AG, Waller DG, Challenor VF, George CF, Amanullah M. The influences of dose and ethnic origins on the pharmacokinetics of nifedipine. Clin Pharmacol Ther 1993; 54: 333, with permission.)

(including benzodiazepines) undergo substantial first pass metabolism by enterocyte CYP3A (CYP3A4 and sometimes CYP3A5).

Recent work has shown that CYP3A4 is the responsible for the back oxidation of reduced-haloperidol to haloperidol and also for the N-dealkylation of haloperidol /59,60/. A negative correlation between clinical response and reduced-haloperidol levels or reduced-halo-

peridol/haloperidol ratios has been observed /61/: hence individuals with higher CYP3A4 activity could respond better to haloperidol than those with lower CYP3A4 activity. In a study on newly hospitalised Chinese patients with schizophrenia, Lane and colleagues found that those who experienced EPS had significantly higher reduced haloperidol concentrations and reduced-haloperidol/haloperidol ratios than the other patients /62/. A trend towards higher haloperidol concentrations was also found in the EPS group. This would be consistent with individuals with lower CYP3A4 activity being more vulnerable to EPS.

CYP3A4 is readily induced by carbamazepine: for most typical antipsychotics twice as much antipsychotic is required to achieve the same plasma concentration in the presence of carbamazepine as in the absence of carbamazepine /48/. This interaction is relevant for the treatment of schizoaffective psychoses. Other substances inhibit metabolism by the CYP3A enzymes (Table 1, modified from /63/). Clozapine toxicity has been reported after the coadministration of erythromycin /64/. Individuals who are CYP2D6 poor metabolisers or who are in receipt of drugs that inhibit CYP2D6 metabolism would be expected to be at increased risk of effects secondary to drug interactions at CYP3A4, and *vice versa*.

TABLE 1

CYP3A inhibitors (modified from /63/)

SSRIs (fluoxetine, fluvoxamine), SNRIs (venlaflaxine, nefazodone)
Steroids (oral contraceptives, prednisolone, tamoxifen, etc.)
Antibiotics (erythromycin, troleandomycin, clarithromycin, isoniazid)
Antifungals (ketoconazole, itraconazole)
AntiHIV drugs (ritanovir, zidovudine)
Analgesics (e.g. dextropropoxyphene)
Anaesthetics (e.g. lidocaine)
Cardiac drugs (nifedipine, verapamil)
Immunosuppressants (cyclosporin)
Cimetidine

3. CYP1A2

3.1 Variation in CYP1A2 activity

CYP1A2 is involved in the metabolism of many typical antipsychotics, clozapine. and olanzapine. A preliminary study showed evidence of interethnic variation, with Japanese showing an increased maximum plasma concentration of olanzapine after a given dose. and a mean half-life of 34 hours as compared with 24 hours in Caucasians /48/. Le Marchand *et al.* /65/ also showed significantly lower CYP1A2 activity in a group of 45 Japanese as compared with 15 Caucasians living in Hawaii. Nakajima *et al.* /66/ showed that CYP1A2 activity as measured by caffeine 3-demethylation was bimodally distributed in a group of 205 Japanese. with 14.1% being poor metabolisers. Other groups have also shown a multimodal distribution of CYP1A2 activity in most /67-70/, but not all /67,69/ populations. Relling *et al.* /71/ showed that CYP1A2 activity was significantly lower in a group of 63 Black subjects as compared with a group of 246 White subjects ($p = 0.036$).

This enzyme is also involved in the metabolism of aromatic and heterocyclic amines, and in most populations is found to be induced by smoking /66-70, 77/. The effect of smoking appears to be absent in Chinese /68/, which has been proposed to be secondary to the lower level of smoking in this ethnic group. Oral contraceptives. postmenopausal replacement oestrogens, and pregnancy appear to reduce CYP1A2 activity /65,73,74/. The lower CYP1A2 activity in women as compared to men appears to be explained by the effect of oestrogens /65/. Le Marchand and colleagues /65/ also found that lutein (which is found in green leafy vegetables) inhibits CYP1A2 activity. Caffeine and paracetamol (acetaminophen) intake increases CYP1A2 activity, as does the consumption of cruciferous vegetables (cabbage, broccoli, Brussels sprouts and watercress). However, the amount of cruciferous vegetable (*e.g.* 500 g broccoli daily for 10 days) required to increase CYP1A2 activity is considerably higher than that commonly present in a normal diet. Intake of meat cooked rapidly at a high temperature has also been shown to increase CYP1A2 activity /75/.

Although environmental factors such as the above are important in contributing towards variability in CYP1A2 activity, Le Marchand and colleagues /65/ found that 73% of the variability remained unexplained after taking into account the major environmental contributors to

the variance in 90 subjects of various ethnic backgrounds in Hawaii. A significant contributor to the variance may be genetic in origin, as suggested by work with inbred mice /76/. A mutation in the promotor region of CYP1A2 has recently been found (Aitchison *et al.*, unpublished data). This mutation shows significant interethnic variation: the frequencies of individuals heterozygous and homozygous for the mutation was found to be 2.3% and 1.1% respectively in 176 Caucasians and 24.4% and 4.1% respectively in 123 Taiwanese.

3.2 Clinical relevance of variations in CYP1A2 activity

The metabolism of clozapine *in vivo* appears to correlate with CYP1A2 activity, although CYP3A4, CYP2C19, and CYP2D6 are also involved /77,78/. Levels of clozapine of at least 350 to 420 ng/ml are associated with therapeutic response /79/, while the incidence of seizures and EEG abnormalities also appears to increase with dose. Elevated plasma clozapine concentrations in Chinese patients have been described (mean steady-state plasma clozapine concentration 60-100% higher than that found in Caucasians, /80/). At the opposite end of the spectrum, very low plasma clozapine levels despite high doses and compliance have been described, in association with very high CYP1A2 activity /81/. It is therefore possible that, analogous to the situation with CYP2D6, there exist individuals with ultrarapid CYP1A2 metaboliser status.

The most important pathways for olanzapine metabolism are CYP1A2, flavin-containing monoxygenase 3, and N-glucuronidation, with minor pathways including CYP2D6 and CYP2C19 /48/. Olanzapine clearance is increased in males (by about 30%) and in smokers, and decreased in the elderly, all of which are consistent with the involvement of CYP1A2. Evidence for the contribution of CYP1A2 to the pharmacokinetics of typical antipsychotics includes the effect of smoking: smoking increases the clearance of fluphenazine and haloperidol by 100% and at least 50% respectively in an affected population.

4. CYP2C ENZYMES

4.1 CYP2C genetics

Four members of the human CYP2C subfamily have been identified: CYP2C8, CYP2C9, CYP2C18, and CYP2C19 /82/: their genes form a cluster at chromosome 10q24. Of these, the role of CYP2C19 in the metabolism of psychotropic drugs has been most extensively studied. Relevant substances include amitriptyline, imipramine, clomipramine, moclobemide, citalopram, diazepam and desmethyldiazepam /83/, as well as clozapine, olanzapine, propranolol, and phenytoin to lesser extents. Four SSRIs (fluoexetine, sertraline, paroxetine, and citalopram) are all able to inhibit CYP2C19 and may also be metabolised by this enzyme. *CYP2C18* lies distal to *CYP2C19* on chromosome 10, is 85.7% homologous to *CYP2C19*, and shows similar substrate specificity towards diazepam /84/, phenytoin /85,86/, and omeprazole /87/. Furthermore, Mamiya *et al.* /88/ found cosegregation of poor metaboliser mutations of CYP2C19 and CYP2C18, indicating that CYP2C18 may not be able to take over from CYP2C19 in individuals deficient in CYP2C19. CYP2C9 was shown by Hashimoto and colleagues /89/ to play a greater role than CYP2C19 in the metabolism of phenytoin, and the Leu^{359} allele, which is present in the heterozygous state in 3.4% of Han Chinese subjects /90/, was seen to be associated with a 40% reduction in the V_{max} for phenytoin.

The incidence of poor metabolisers (PMs) of CYP2C19 in different populations has been reviewed /23,91-93/. There is substantial interethnic variation: the frequency of PMs is 2-5% in Caucasians, 2% in Saudi Arabians, 4% in Black Zimbabweans, 5% in Ethiopians, 13% in Koreans, 15-17% in Chinese, 21% in Indians, and 18-23% in Japanese. Indeed, when the square root of the PM frequency (representing the total frequency of mutant *CYP2C19* alleles) was plotted versus longitude, an increase in this value versus longitude was seen, with a step in the value occurring somewhere between Saudi Arabia and Bombay (Fig. 3).

There are two wild-type *CYP2C19* alleles (*CYP2C19*1A* and *CYP2C19*1B*), and seven defective alleles which are responsible for the PM phenotype /93/. The most common defective allele is *CYP2C19*2A* (a $G_{681}A$ substitution in exon 5, which creates an aberrant splice site, previous name for this allele, *m1*). A variant of this allele, *CYP2C19*2B*, contains a $G_{276}C$ substition in exon 2 which creates a

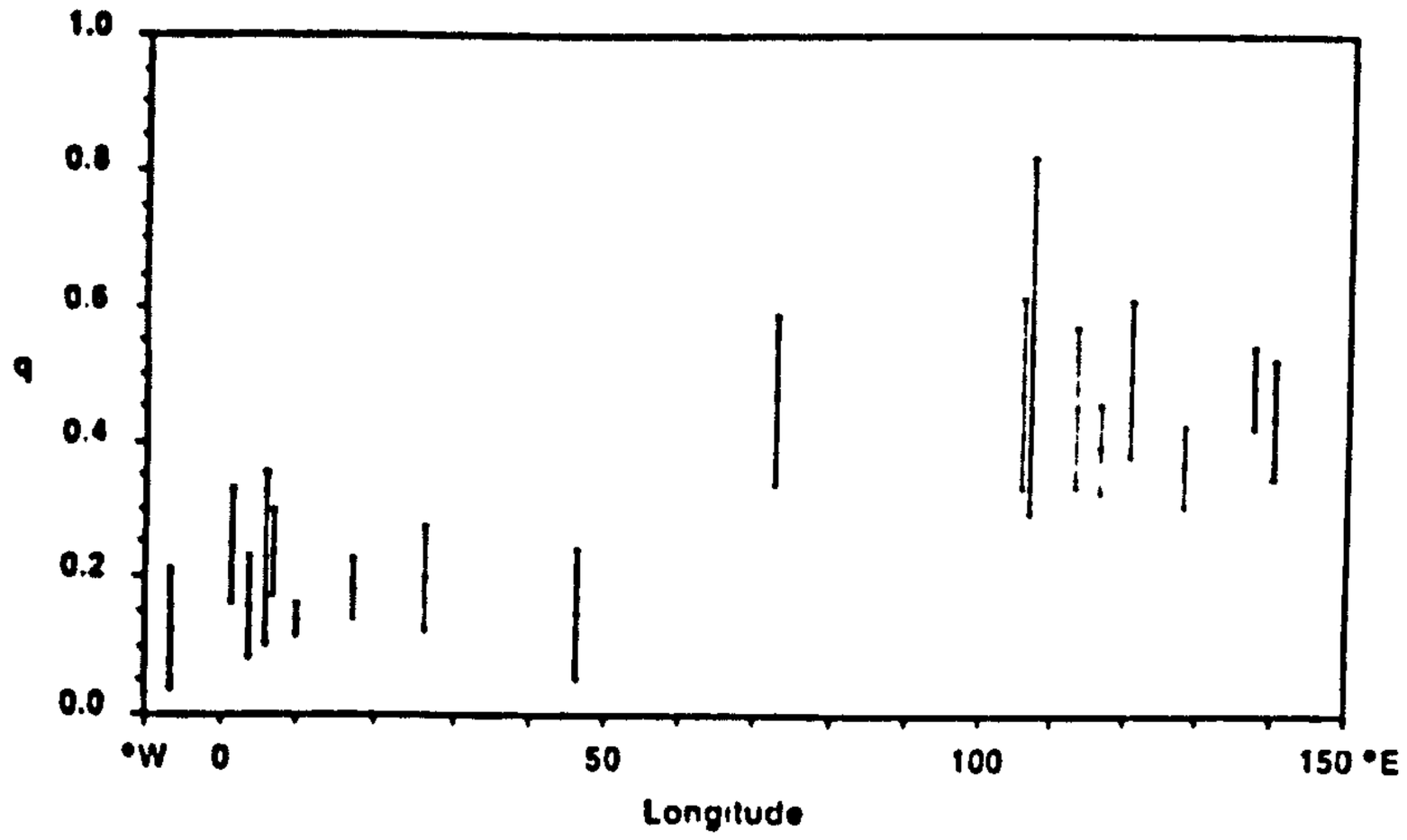

Fig. 3: Estimates of q, the total frequency of mutant CYP2C19 alleles, with 95% confidence limits in relation to longitude. (From: Price Evans DAP, Krahn P, Narayanan N. The mephenytoin (cytochrome P450 2C19) and dextromethorphan (cytchrome P450 2D6) polymorphisms in Saudi Arabians and Filipinos. Pharmacogenetics 1995; 5: 70, with permission.)

$Glu_{92}Asp$ change; this allele comprises 15% of the *CYP2C19*2* allele in Caucasians, but was not observed in 53 Japanese *CYP2C19*2* alleles studied. The two CYP2C19*2 alleles account for 86% of PM alleles in Caucasians and 69-87% in Orientals. The second major defective allele is *CYP2C19*3* (a $G_{636}A$ mutation in exon 4, which creates a premature stop codon, previous name for this allele, *m2*); this comprises 13-31% of PM alleles in Oriental populations and 1.5% in Caucasians. A third PM allele, *CYP2C19*4* (an A→G mutation in the initiation codon), accounts for 3% of Caucasian PM alleles. *CYP2C19*5* (a $C_{1297}T$ mutation in exon 9 which results in an $Arg_{433}Trp$ change in the haem binding region) accounts for 1.5% of Caucasian PM alleles and is rare in Orientals. *CYP2C19*6* (a $G_{395}A$ base substitution resulting in an $Arg_{132}Gln$ coding change in exon 3) and *CYP2C19*7* (a GT→GA mutation in the donor splice site of intron 5) each account for a further 1.5% of Caucasian PM alleles. *CYP2C19*8* (a $T_{358}C$ substitution resulting in a $Trp_{120}Arg$ change in exon 3) is a newly characterised, rare defective allele. The products of *CYP2C19*6* and *CYP2C19*8* show reduced catalytic activity (2% and 9%

of wild-type S-mephenytoin hydroxylase activity, respectively); the other mutants are associated with failure to express active CYP2C19. *CYP2C19*2A* and *CYP2C19*3* have both been identified in an Ethiopian population, and found to account for all the PM alleles in the 114 individuals studied /92/.

4.2 Clinical studies of CYP2C19

Omeprazole has been used as a probe drug in studies of CYP2C19 activity. In single-dose studies the clearance of omeprazole has been found to be higher in CYP2C19 EMs than PMs in Caucasians, Chinese, and Koreans, with the clearance in Caucasian EMs being significantly higher than that in both Chinese and Korean EMs /91/. After multiple doses of omeprazole, the mean areas under the plasma concentration-time curve for the parent drug indicated that heterozygous individuals had a reduced rate of metabolism as compared to homozygous EMs. It has therefore been hypothesised that the difference in clearance between Caucasians and Orientals is due to the relatively high proportion of heterozygous EMs among Orientals as compared with Caucasians.

In the case of diazepam, the clearance is significantly lower in Caucasian and Korean CYP2C19 PMs than EMs /91/. However, in Chinese, no significant difference between the elimination half-life of eight EMs and eight PMs was found, and the mean clearance in the whole group was relatively low as compared to Caucasians. It has been suggested that among the eight Chinese EMs, seven with a relatively low diazepam clearance might be heterozygous, which would explain the low overall clearance and the lack of significant difference between the EM and PM groups. Alternatively, differences in the contribution of CYP3A4 to diazepam pharmacokinetics in the different ethnic groups could explain the different findings. Like CYP2D6, CYP2C19 often functions as a high-affinity, low capacity enzyme, which is more important at low drug doses. With higher doses, multiple-dosing, or in the case of CYP2C19 deficiency, CYP3A4, which has a relatively high capacity and often shows relatively low substrate affinity, increases in its contribution to overall drug clearance. Schmider *et al* /94/ have calculated that even with single doses, approximately 60% of diazepam clearance is CYP3A4-dependent. The relatively high incidence of low CYP3A4 activity in Chinese may therefore contribute to the low mean diazepam clearance,

and, if polymorphisms in CYP3A4 and CYP2C19 do not cosegregate, could contribute towards the lack of a significant difference between diazepam clearance in S-mephenytoin PMs and EMs. It has been noted that "many Hong Kong physicians routinely prescribe smaller diazepam doses for Chinese than for white Caucasians" 95/; this tradition is consistent with the lower clearance found experimentally.

5. DRUG-DRUG INTERACTIONS

When prescribing psychotropic drugs that are subject to interethnic variations in metabolism, it is important to remember that interactions with non-psychotropic drugs at these sites may occur. A summary of substrates metabolised by the enzymes discussed above is listed in Table 2. Many enzymes show overlapping substrate specificity, and only the major routes of metabolism are shown. It should also be remembered that substances may exert considerable inhibitory effect at a given route, without being metabolised by that enzyme (*e.g.* quinidine in the case of CYP2D6). An individual who is a PM of a particular CYP enzyme will tend to be more susceptible to drug-drug interactions at the other cytochromes.

6. CONCLUSIONS

There is considerable interethnic variability in the activity of the DMEs. Although some studies aiming to show correlations between the activity of a single DME and clinical effects have yielded conflicting results, this may be because of the existence of alternative metabolic pathways using other DMEs. For example, in the case of CYP2D6 or CYP2C19 deficiency, CYP3A4 will often be able to play a substitutive role. Individuals who have a relatively low CYP2D6 or CYP2C19 activity will therefore be more susceptible to the effects of CYP3A4 inhibitors. Furthermore, it would be logical to hypothesise that while the clinical effects of single enzyme deficiency might not be consistent, the effects of deficiency of more than one enzyme might well be significant. This hypothesis is supported by the analagous finding of Rojas and colleagues /97/ that smokers with combined CYP1A1 and glutathione S-transferase M1 (GSTM1) deficiency showed significantly higher levels of activated DNA-bound potentially

TABLE 2

Some substrates of polymorphic CYP enzymes /83,94,96/

CYP2D6		CYP3A4			CYP1A2	CYP2C19	CYP2C9
Haloperidol	Codeine	Haloperidol	Carbamazepine	Progesterone	Chlorpromazine	Clozapine	Amitriptyline
Perphenazine	Dextromethorphan	Clozapine	Valproate	Oral	Trifluoperazine	Olanzapine	Zopiclone
Zuclopenthixol	Methadone	Sertindole	Codeine	contraceptives	Clozapine	Amitriptyline	Theophylline
Thioridazine	Methamphetamine	Amitriptyline	Dextromethorphan	Cortisol	Olanzapine	Imipramine	Phenytoin
Risperidone	Methylenedioxymeth-	Imipramine	Dextropropoxyphene	Prednisolone	Amitriptyline	Clomipramine	Tolbutamide
Sertindole	amphetamine*	Clomipramine	Orphenadrine	Amiodarone	Imipramine	Moclobemide	Warfarin
Amitriptyline	Propranolol	Fluoxetine	Erythromycin	Diltiazem	Clomipramine	Citalopram	
Clomipramine	Metoprolol	Fluvoxamine	Clarithromycin	Nifedipine	Zopiclone	Diazepam	
Imipramine	Pindolol	Sertraline	Doxycycline	Nimodipine	Tacrine	Propranolol	
Desipramine	Timolol	Nefazodone	Isoniazid	Nicardipine	Caffeine	Phenytoin	
Nortriptyline	Flecainide	Trazodone	Rifampicin	Digitoxin	Theophylline	Ibuprofen	
Fluvoxamine	Mexiletine	Venlaflaxine	Trimethoprim	Proguanil	Aminophylline	Diclofenac	
Paroxetine	Perhexiline	Diazepam	Testosterone	Quinidine	Paracetamol	Naproxen	
Mianserin	Propafenone	Midazolam	Androsterone	Cisapride	(Acetaminophen)	Omeprazole	
Desmethyl-	Metoclopramide	Clonazepam	Dapsone	Lidocaine	Zopiclone	Pantoprazole	
citalopram	Orphenadrine	Alprazolam	Dehydroepiandro-	Terfenadine		Proguanil	
Maprotiline	Ondansetron	Zolpidem	stendione	Cyclosporin		Piroxicam	
Venlaflaxine		Caffeine	Oestradiol	Ondansetron			
		Theophylline	Tamoxifen	Vinblastine			

*also known as "ecstasy"

carcinogenic metabolites than individuals with CYP1A1 or GSTM1 deficiency alone. We have noted that there is a lower population mean CYP2D6 activity, lower CYP1A2 activity, and higher incidence of CYP2C19 poor metabolisers in Japanese as compared to Caucasians. Similarly, a lower population mean CYP2D6 activity and a much higher incidence of a mutation in the promotor of CYP3A4 has been found in Black subjects, while impaired CYP3A4 activity and a high frequency of CYP2C19 poor metabolisers has been found in individuals from the Indian subcontinent. We would therefore suggest that future studies focussing on the relevance of ethnic influences in pharmacogenetics to the treatment of psychosis should encompass methodology that is capable of analysing the variation in activity of all the DMEs (including the role of dietary and other environmental factors such as smoking) relevant to the population being studied. There is a paucity of studies addressing ethnic variation in pharmacodynamic factors; this issue should also be addressed in future work.

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